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# Electric-field Driven Sample Clean-up Strategies for Mass Spectrometry Applications

by

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## ABSTRACT

Sodium dodecyl sulfate (SDS) is commonly used for protein solubilization prior to tryptic digestion, while inorganic salts, with SDS, are typically used during the extraction of macromolecules from biological samples in bottom-up proteomics. Above a certain threshold, the presence of inorganic salts and/or SDS interferes with electrospray ionization-mass spectrometric (ESI-MS) analysis of peptides and digested proteins. Sample preparation strategies have been developed to circumvent this issue. This work describes electric-field driven techniques for the removal of inorganic anions and SDS from relatively high conductivity and micellar sample solutions in microliter volumes of peptides and digested protein samples. The one-step inorganic anion removal process is based on controlling the apparent electrophoretic velocities of anions at a boundary that separated the sample and the acidic extraction solution (ES) in a fused-silica capillary. Removal of >80% inorganic anions and >80% analyte signal restoration were achieved. The applicability of the method was also extended to the removal of chloride ions prior to the analysis of low levels of arsenic and vanadium using inductively coupled plasma MS. The dodecyl sulfate ( $\text{DS}^-$ ) as sodium salts were electrokinetically removed offline using an acidic ES containing acetonitrile in a fused silica capillary dipped into the sample. The high amount of organic solvent collapses the micelle-peptide complex, releasing the peptide and thus is amenable to efficient removal of  $\text{DS}^-$  from the sample solution. Percent MS signal intensity restoration of 74-83% and 89-95% percent MS signal intensity reduction of  $\text{DS}^-$  were attained. Lastly, an online  $\text{DS}^-$  removal system directly connected to the MS instrument was developed. The removal of  $\text{DS}^-$ , and the retention and subsequent analysis of peptides were performed using an acidic ES with 40% acetonitrile. The mechanism was similar to the offline strategy and resulted in the high improvement of ESI-MS signals of peptides.

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### **List of abbreviations**

BSA	bovine serum albumin
CE	capillary electrophoresis
DNA	deoxyribonucleic acid
DT	direct tissue trypsinization
DTT	dithiothreitol
eFASP	enhanced filter-aided sample preparation
EOF	electroosmotic flow
ESI	electrospray ionization
FAB	fast atom bombardment
FASP	filter-aided sample preparation
FFPE	formalin-fixed paraffin- embedded
GSF	gas phase fractionation
H-BN	hexagonal boron nitride
HDL	hydrophilic-lipophilic balance
HLB	hydrophilic lipophilic;
HMEC	human mammary epithelial cell
HPLC	high performance liquid chromatography
ICP	Inductively-coupled plasma
IEF	isoelectric focusing
IMAC	immobilized metal affinity chromatography
ISD	in-solution digestion
LC	liquid chromatography
LTQ	linear trap quadrupole

MALDI	matrix-assisted laser desorption/ionization
MEKC	micellar electrokinetic chromatography
MIP	molecularly – imprinted polymer
MS	mass spectrometry
MSS	micelle-to-solvent stacking
PAGE	polyacrylamide gel electrophoresis
PTM	posttranslational modification
QToF	quadrupole time-of-flight
RNA	ribonucleic acid
RPLC	reverse phase liquid chromatography
SCX	strong cation exchange
SDS	sodium dodecyl sulfate
SPE	solid-phase extraction
UVA	ultraviolet A
WCX	weak cation exchange

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## **List of Included Publications**

### ***Peer-reviewed Articles***

1. R.M. Tubaon, P.R. Haddad, J.P. Quirino, Sample clean-up strategies for electrospray ionization mass spectrometry applications in bottom-up proteomics: Trends from 2012–2016. *Proteomics*. DOI: 10.1002/pmic.201700011. (Chapter 1)
2. R.M. Tubaon, P.R. Haddad, J.P. Quirino, One-step selective electrokinetic removal of inorganic anions from small volumes and its application as sample clean-up for MS techniques. *J. Chromatogr. A*, 2017, *1488*, 134-9. (Chapter 2)

### ***Oral Presentations***

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## Chapter 1

### Introduction and Literature Review

#### 1.1 Proteomics: Definition and aims

The essential role of proteins in supporting life has long been recognized since the early stages of biological research. The word protein, which is derived from the Greek word *proteios*, meaning “the first rank or position” was first used in the early 1800s to demonstrate the importance of proteins and its functions <sup>1</sup>. Around 9000 specialized protein types per nucleated cell regulate the multivariate function of each specific cell <sup>1,2</sup>. The discovery that DNA possesses all necessary information to create an organism led to the idea of the central dogma of molecular biology, which is the one-directional flow of information from DNA to RNA, and finally to protein. Briefly, transcription occurs in the nucleus and uses DNA as a template to make RNA. RNA nucleotides are also utilized as building blocks. Translation, on the other hand, occurs in the cytoplasm and uses mRNA as a template to make protein. Amino acids are used as building blocks in translation processes.

The innovations of DNA microarray technology <sup>2,3</sup> and serial analysis of gene expression (SAGE) <sup>4</sup> allowed the analysis of mRNA expressions. The transcriptome data provide information about the degree of gene activity in individual tissues and relationship to cell function, development stage, response to stimuli, and disease. However, this information does not fully reflect the protein content of the cell. Studies show that the information based on the mRNA is only one step of the long sequence of events in the synthesis of proteins <sup>2,5-6</sup>. Generated proteins are subjected to posttranslational modifications (PTMs). In addition, different types of proteins are formed from one gene, i.e two in bacteria, three in yeast, and three or more in humans <sup>5,6</sup>. Elucidation of mechanisms of disease, aging, and effects of environment, and the behavior of gene products is very challenging by studying the genome

alone <sup>7</sup>. Proteomics provides a direct approach to investigate the level of production, as well as the activity or degree of modification of the products of our genetic codes.

The term proteomics was first coined by Wilkins in the 1994 <sup>8</sup> and is defined as the global analysis of the set of proteins, called proteome, produced in an organism, cell line, tissue, or biological context at a given time. Proteomics encompass quantification of protein abundance and expression, identification of protein-protein interactions, detection of PTMs, and turnover as a function of time, space, and cell type, among others. The proteome is fundamentally dynamic than the genome or mRNA complement found within the cell. The physical and chemical diversity of one protein molecule to the next makes the analysis of proteome a big challenge for scientists.

## **1.2 Methodologies of proteomics**

### ***1.2.1. Separation and isolation of proteins***

#### **1.2.1.1. 1-and 2-D gel electrophoresis.**

Prior to the identification of peptides and proteins, the protein or peptide molecules in the sample solution needed to be separated. Separation of proteins had been demonstrated in 1968 when Kendrick & Margolis presented 2-dimensional gel electrophoresis by combining native isoelectric focusing with pore gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for serum protein mixtures <sup>9, 10</sup>. O'Farrell <sup>11</sup>, Klose <sup>12</sup>, and Scheele<sup>13</sup> mapped proteins of *Escherichia coli*, mouse, and guinea pig, respectively, using 2-dimensional gels in 1975.

One-dimensional gel electrophoresis is one of the methods in separating proteins in a mixture and is based on molecular masses of the proteins. This method is generally applied to resolve proteins of molecular masses from 10 – 300 kDa.

For crude and more complex samples, 2-D gel electrophoresis is employed. This method is also used to separate proteins that have PTMs. In this separation step, the proteins

are separated based on two properties, the net charge at a set of pH and molecular mass. In the first dimension, proteins are separated by isoelectric focusing. The overall charge of the proteins is a result of reaction of functional groups to the pH of the solution. The net charge of a protein can be zero, which is called the isoelectric point, by manipulation of the ambient pH. In the presence of an electric field, and when there is a pH gradient embedded within the gel strip, the proteins migrate to a place where their pI equals zero. In the second dimension, the proteins are separated by the differences in their molecular masses by SDS- PAGE. The separation is carried out using buffers, most commonly used is Laemmli buffer, and can separate from 10 to 300 kDa molecular masses. The appropriate gel to use is dependent on the molecular mass of the proteins <sup>14, 15</sup>. After separation, the proteins can be visualized by universal or specific staining methods.

Since their inception, 1-D and 2-D gel electrophoreses are the most preferred and most effective methods to resolve protein mixtures. Both stages, however, are slow and tedious procedures, and cannot be easily automated. One 2-D gel electrophoresis can take up to two days to complete, and only one sample can be loaded into the gel. Because of these limitations, a number of approaches have been developed to bypass protein gel electrophoresis. One major innovation is the introduction of bottom-up proteomics, which is the conversion of an entire protein mixture to peptides by digestion. More information on bottom-up proteomics will be presented in Section 1.3.1.

### ***1.2.2. Acquisition of protein structural information***

The final step for protein analysis is the identification of specific proteins and peptides, as well as the quantification of protein expressions. The determination of peptides and proteins was pioneered by the Edman degradation method, and was followed by the introduction of mass spectrometry. The results are compared to data repositories, the most common being the World-2DPAGE database.

#### 1.2.2.1. Edman degradation

With the success of the separation and visualization of the proteins, the identification of the proteins remained very challenging. The lack of sensitive protein sequencing technology was the biggest obstacle that researchers encountered. The major development on the identification of peptides and proteins occurred when sequencing of proteins by Edman degradation was introduced <sup>16</sup>. This protein sequencing method is an automatic determination of amino acid sequences in peptides and proteins and is based on the principle of the phenylisothiocyanate reaction <sup>16,17</sup>. Under mild alkaline conditions, the uncharged N-terminal group is reacted with phenylisothiocyanate to form a cyclical phenylthiocarbamoyl derivative. This derivative is cleaved as a thiazolinone derivative under acidic conditions and then is selectively extracted into an organic solvent and treated with acid to form phenylthiohydantion-amino acid. In theory, this procedure can be applied for degradation of any amino acid sequence, regardless of length. However, in practice, peptides comprising up to 40 amino acids can be degraded <sup>18</sup>. Long peptide chains have poor solubility in the degradation media, in addition to the unreaction of modified N-terminal residue to the Edman reagent. Cleaving long peptide chains into shorter peptides prior to the degradation process can circumvent this drawback.

The use of Edman degradation is not as popular now as a few decades ago because of the invention of mass spectrometry (MS) for protein analysis. However, Edman degradation is still used by some researchers in the absence of MS instrumentation, most especially in analyzing relatively abundant proteins. In addition, this degradation method is also applied when determining the true start of the N-terminal sequence of a protein <sup>18</sup>.

#### 1.2.2.2. Mass spectrometry.

The innovation of mass spectrometry (MS) is one of the most important developments for proteins and peptide identification. The MS instrument distinguishes charged molecules in the

gas phase based to their mass-to-charge ratio. Electric or magnetic fields are generated inside the MS instrument. An MS instrument is composed of an ionization source, analyzer, detector, data processor, and vacuum pumps.

Before any separation by MS, molecules must be ionized and converted into the gas phase using different techniques. Prior to the 1980s, electron ionization was the most prevalent ionization source of MS instruments. However, this ionization source is only useful for small molecules below the mass range of proteins. Other researchers employed fast atom bombardment (FAB) for the analytical and structural studies of proteins<sup>19, 20</sup>. However, FAB analysis requires large volumes of samples and can only be employed for analysis of intact proteins, and this made FAB unsuitable for protein or peptide analysis in biological samples. Attempts to transform large polar organic molecules into gas-phase ions to extend the sensitivity, applicability, and accuracy of MS had been very challenging.

The advent of soft ionization techniques, namely electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), in the late 1980s effectively convert large molecules into gas phase without affecting the integrity of the molecules. The development of MALDI and ESI made the acquisition of mass spectra of proteins and peptides for minute quantity sample solutions possible. MALDI utilizes a light – absorbing matrix mixed with the sample solution in an appropriate solvent on the sample probe. The matrix compound is typically 10 000-fold molar in excess. The solvent is then evaporated, leaving the co-crystallised analyte molecules behind. The sample probe is subjected to a short pulse of laser of a specific wavelength. The matrix partially vaporizes and carries the intact analyte molecules into the gas phase<sup>21</sup>. Negatively and positively charged analyte molecules are formed through the exchange of protons between the analytes and the matrix molecules<sup>22</sup>. MALDI is more tolerant than ESI when analyzing samples with contaminants like buffers, salts, or even as high as 2 M urea, however, reproducibility is a constant challenge.

### *Electrospray ionization (ESI)*

ESI emerged in the late 1980s as the softest ionization technique for large and complex species in a solution. It was Fenn's work in ESI that revolutionized the modern day technique of ESI-MS and its application in biological macromolecules <sup>23</sup>. The transfer of ionic species from the sample solution to the gas phase in ESI generally involves three steps, namely: (1) electrospray nebulization or the formation of a mist of charged droplets; (2) droplet disintegration and solvent evaporation; and (3) ion ejection from the highly charged droplets.

During electrospray nebulization (1), the sample solution is introduced into a capillary tube, where a high electric field located at the tip of the tube produces an electric gradient on the fluid. When the electrostatic repulsion is greater than the surface tension, an electrical spray of charged, small drops leaves the surface and travels towards through the surrounding gas to the counter-electrode. The dispersion of a liquid into electrically charged droplets is a combination of droplet formation and droplet charging <sup>24</sup>. Droplet formation, on one hand, is greatly influenced by the liquid's flowrate, surface tension, and the electrolyte concentration<sup>24</sup>. An increase of any of these variables causes difficulties for the electric field to produce the desired charged aerosol for MS. Increasing the electric field to overcome the adverse effects of increasing any of the mentioned variables may give rise to electric discharge, which is problematic in the formation of negatively charged droplets. Modifications of simple electrospray system, such as the dilution of the aqueous solution by the coaxial addition of sheath liquid (e.g. methanol, acetonitrile, ethanol, isopropanol, or 2-methoxyethanol) and utilization of pneumatic nebulization, are aimed at increasing tolerance towards adverse effects of high liquid flowrate, high surface tension, and high electrolyte concentration. Droplet charging and the mechanism of its formation, on the other hand, is a matter of design or particular constraints of the spray capillary, whether at a high voltage or at ground potential. When the capillary is at a more positive potential than the counter-electrode, positively



charged droplets will result. A reversal of the electric field will result in the production of negatively charged droplets<sup>24</sup>.

Droplet disintegration (2) results when there is a sufficient droplet deformation and when the surface tension is exceeded by the charge density electrostatic repulsion<sup>24</sup>. When droplets leave the liquid front at the tip of the spray capillary, the electrostatic repulsion is greater than the cohesive forces that hold the liquid together. In the gas phase, at atmospheric pressure, the size of the droplet reduces due to the evaporation of the solvent, and results to the increase of the charge density at the surface of the droplet. In addition, shear forces affect the droplets during their flight through dense gas. The increase in charge density and the shear forces on the droplets causes the droplets to undergo deformation. High electric fields are localized at the protrusions on the surface of the droplets. Droplets become unstable and disintegrate.

Naked sample ions are ejected (3) from the surface of the droplets when the electric field at the surface becomes sufficiently high. This process is called ion evaporation. These naked sample ions are then taken into the mass spectrometer, and the ion formation process is insignificant during ESI-MS. Incompletely desolvated ion can be desolvated by the dry curtain gas in the heated ion sampling tube or by collision inside the vacuum<sup>25, 26</sup>.

ESI is well known for producing multiply charged species of larger molecules. This phenomenon of ESI is more advantageous than MALDI because multiple charging makes the instrument amenable for the elucidation of very large molecules.

### **1.3. Mass spectrometry-based proteomics**

In recent years, MS-based protein identification has matured immensely through advances in technology, instrumentation, sample preparation, and computational analysis<sup>27</sup>. Analyses using MS have been commonly used because of better sensitivity and throughput compared to other techniques, such as cell imaging and protein arrays. There are generally three MS-based

approaches practiced in proteomics, namely; top-down, middle-down, and bottom-up, as depicted in Figure 1.3.1.

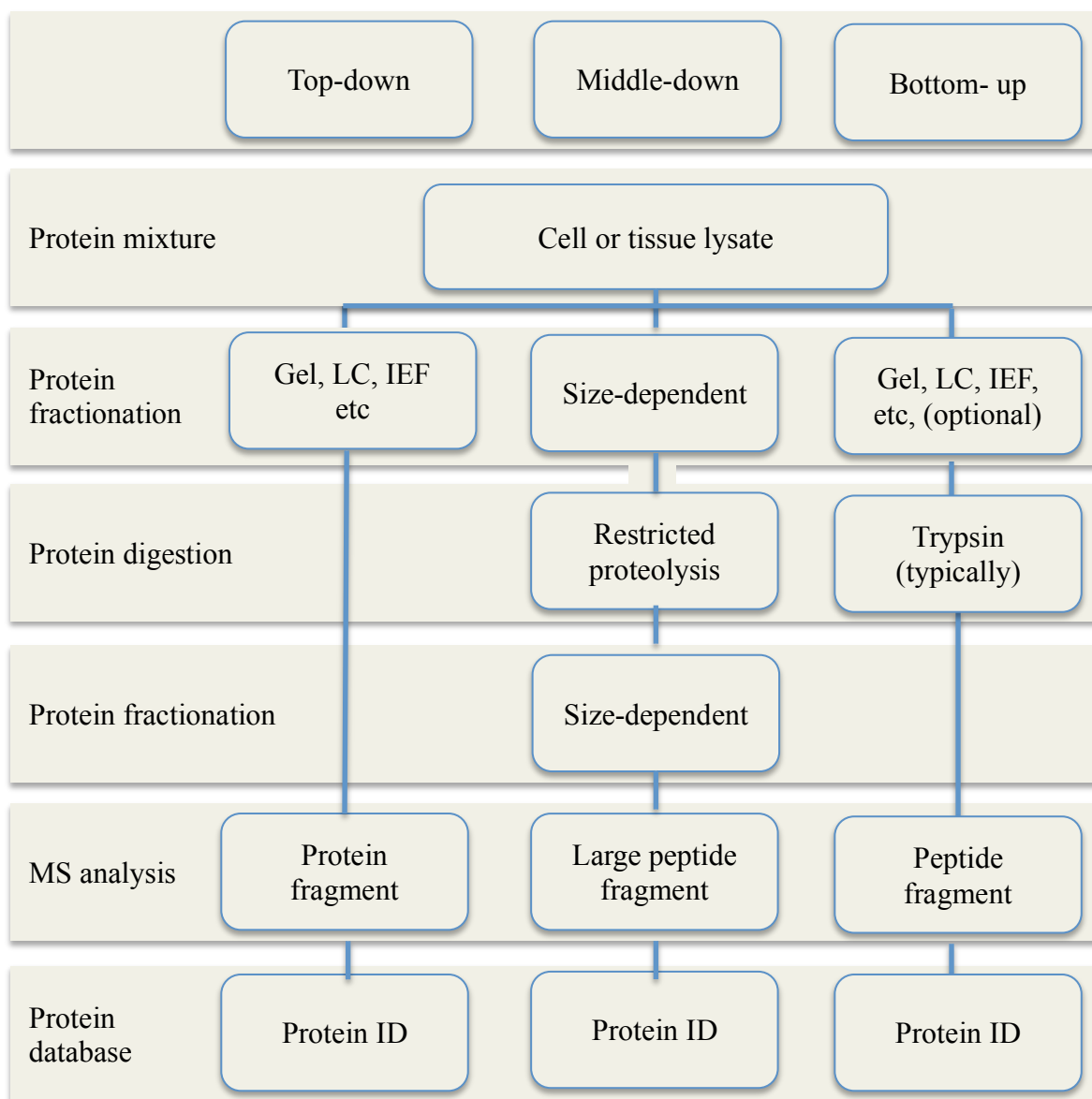


Figure 1.3.1. Bottom-up, middle-down, and top-down MS-based proteomics workflow. In the top-down approach (left), intact proteins are directly analyzed in the MS. In the middle-down approach (middle), the proteins are digested normally using Glu-C or Asp-N producing large peptide fragments. In the bottom-up approach (right) proteins are generally digested with trypsin resulting to shorter set of peptides/peptide fragments. Protein identification can be done using protein databases, gene-based databases, or de novo sequencing, as examples.

The elucidation of intact proteins is referred to as a top-down approach in proteomics. The proteins are fractionated by gel electrophoresis or liquid chromatography (LC). These intact proteins are introduced into the MS instrument in the gas phase by ESI. The analytes are fragmented and the molecular masses of both protein and fragment ions are produced. A complete description and identification of the proteins results when a sufficient amount of informative fragment ions is observed<sup>28</sup>. Formidable challenges remain to be overcome with top-down approach. The production of extensive gas-phase fragmentation of intact protein ions, especially for large proteins, has long been the major hurdle of implementing top-down approach in proteome research, along with sample fractionation, MS fragmentation, and spectral interpretation. Extensive researches were performed to circumvent this issue. Han and co-workers<sup>29</sup> successfully used electrospray additives, heated vaporization, and separate noncovalent and covalent bond dissociation to extend the molecular masses to be analysed by MS to 200 kDa. A high amount of energy was utilized into the ionized proteins in the ion injection and collision dissociation steps. When compared to bottom-up proteomics, however, there is no loss of important information in the reduction from the intact protein to collection of peptides in a top-down approach<sup>30</sup>. Assembling sequences of peptides to identify the protein in the sample is apparently not necessary in top-down proteomics.

An emerging methodology that combines the strengths of both top-down and bottom-up approaches is the so-called middle-down proteomics. In this approach, peptides that are longer than the products of tryptic digestion (0.5 – 3 kDa on average) result from limited digestion or restricted proteolysis. Because the proteins are cleaved in a way that longer peptides are produced, valuable sequence information can be derived, such as the possibility of mapping information after PTMs of the proteins. In addition, ease in pre-fractionation by most LC methods is also made possible by the resulting peptide chains in middle-down approach. Intensive research has been undertaken to find suitable proteases to generate longer

peptides, as well as MS techniques to analyse them<sup>31-33</sup>. Limited proteolysis using trypsin was also used to produce longer peptides, but a more complex mixture of short and long peptides may result. High-throughput demonstrations of middle-down approach are not yet often implemented because of the challenge of obtaining long peptides within the 3-15 kDa range. So far, middle-down proteomics has been used for the study of histone PTMs<sup>34</sup> and ribosomal proteome<sup>32</sup>.

### ***1.3.1. Bottom-up approach***

The bottom-up approach is the most widely used MS-based workflow in identifying proteins and PTMs and is the MS characterization of peptides from enzymatic digestion of proteins<sup>35, 36</sup>. Tryptic digested peptides are readily solubilized and separated compared to solubilization and separation of intact proteins, as in top-down approach. In addition, digested peptides are easily analyzed by MS, thus providing useful and sufficient information in the identification of the parent protein.

Depending on the type of sample at hand, extraction and isolation of proteins from other biomolecules is required as the first step in bottom-up proteomics. The physical and chemical interactions of the proteins with other biomolecules from their molecular subcompartments make the isolation and analysis of the proteins of interest by LC-MS very challenging. After isolation of proteins from the rest of the biomolecules in the sample, typically, proteolytic digestion of the intact protein is performed. Briefly, a typical protocol for proteolytic digestion of proteins with trypsin, most commonly used protease in protein digestion, initially involves denaturation of the proteins using chaotropic agents, such as SDS or urea. This is followed by the reduction of disulfide bridges using dithiothreitol (DTT), and alkylation of the cysteines using iodoacetic acid or iodoacetamide<sup>37</sup>. Trypsin is a serine protease which cleaves at the carboxyl side of arginine and lysine with the exception when lysine and arginine is N-linked to aspartic acid. This results in peptides with an average length

of ~14 amino acids having at least two positive charges <sup>37</sup>. During digestion, adequate solubilization and unfolding of all proteins in the mixture are crucial for the protease to gain access to cleavage sites. The use of protein solubilizers, the most widely used of which is SDS, has improved digestion efficiency. SDS is a suitable chaotropic agent for solubilisation because it denatures and disrupts macromolecules efficiently. Binding of the nonpolar end of SDS to side chains or tails of the protein results in effective denaturation <sup>37</sup>. The digestion is performed at slightly basic pH (pH 8) in ammonium bicarbonate at 37°C and may take up to 18 h, depending on the way the digestion is performed. The digestion is stopped by an addition of an acid, normally, formic acid.

Selective proteolytic digestion in bottom-up proteomics decreases the complexity of a highly heterogeneous mixture of proteins with diverse physicochemical properties. This process circumvents the challenges associated with intact protein analysis, most especially in ionization and MS characterization. In this strategy, the peptides formed after digestion are within the range ~500 – 3000 Da. Because the peptides produced are shorter, separation by LC is more efficient, and sensitivity is better due to lower molecular mass and fewer charge states <sup>38</sup>.

#### **1.4. Sample cleanup**

Sample preparation is a vital step prior to MS analysis, such as in ESI-MS, and can greatly affect the proteome data downstream in the analysis. While the use of SDS is important in bottom-up proteomics, SDS is detrimental to ESI-MS peptide analysis. Dodecylsulfate (DS<sup>-</sup>) signals dominate the spectra, suppressing the peptide/analyte signals <sup>39, 40</sup>. The mechanism of signal suppression caused by DS<sup>-</sup> is described by Rundlett and Armstrong <sup>41</sup>. The suppression caused by the surfactant in ESI-MS is due to the Coulombic interaction between oppositely charged analytes and the surfactant ions at the surface of the ESI-produced droplets. It has been reported that surfactant ion concentration above 10<sup>-5</sup> M causes severe signal suppression

when the analysis is done via direct infusion <sup>42</sup>. As for most bottom-up proteomic analysis, which is primarily through LC-ESI-MS, an SDS level of up to 0.01% is well-tolerated <sup>40</sup>.

Buffers and inorganic salts are also present in the biological mixture or are added during sample preparation. Typically, macromolecules are extracted from the biological samples using significant concentrations of nonvolatile buffers and salts. High concentrations of inorganic salts, such as sodium chloride present in biological samples, are unfavorable in ESI-MS due to a matrix effect that causes signal suppression or signal enhancement <sup>43</sup>. Many researchers have been investigating the ionization suppression phenomenon for complex biological samples. Most of these works have concluded that the matrix effects are caused by the ionization competition of different species in the ESI-MS instrument <sup>44, 45</sup>, while others have presented evidence that suppression is caused by competition of co-eluted species with the analyte of interest <sup>46</sup>. This undesirable phenomenon is generally not reproducible or repeatable between samples and thus, compromises the integrity of the analysis.

Therefore, strategies for depletion of SDS and inorganic salts pre-and/or post-digestion are continually being developed to enable an efficient and reproducible analysis of samples to ESI-MS. In many cases where sodium ions are left in the sample solution after clean-up, these ions form adducts with peptides or proteins in ESI-MS <sup>47</sup>. This process is a common occurrence in ESI-MS and the metal ion adducts formed often have higher sensitivities than protonated or deprotonated molecules. Consequently, metal adduction is advantageous in quantification, most especially for low concentration analytes <sup>48</sup>. Algorithms have been established to determine the peptide or protein represented by the adducts in positive and negative mode in ESI-MS <sup>49</sup>. The following section describes the desalting and SDS removal strategies for sample clean-up used for bottom-up proteomics prior to ESI-MS analysis.

Inorganic salt (desalting) and SDS removal are most significant clean-up protocols prior to the analysis of peptides in ESI-MS for bottom-up proteomic workflows. Desalting, on one

hand, may include solid-phase extraction (SPE), and membrane-based desalting methods such as dialysis, filtration, and electro-filtration/dialysis. SDS removal, on the other hand, may include SPE, membrane-based such as filter-aided sample preparation, precipitation using organic solvents and buffers. Briefly, the mentioned techniques will be described in the following sections.

#### **1.4.1. Desalting**

##### **1.4.1.1. Solid-phase extraction (SPE) desalting methods**

SPE is a widely used technique in sample preparation and has been thoroughly discussed in numerous reviews <sup>50-52</sup>. Generally, an aqueous sample solution is passed through an immobilized phase and then the retained analytes are eluted with an appropriate solvent or a mixture of solvents, or by thermal desorption into the gas phase. The intermolecular interaction characteristics of the phases influence the retention and elution of the analytes. Low chromatographic efficiencies caused by the relatively large sample volume used, insufficient retention, together with the need for multiple steps and extensive investment of time, limit the application of SPE. These disadvantages are more pronounced when analyzing biological systems, wherein the amount of sample is very limited and the use of SPE may result in loss of analyte and low sample throughput. Through the years, various sorbents and sampling formats had been studied and developed to circumvent these limitations. The type and nature of the analytes dictate the kind of sorbents used in the SPE mode. Sorbents being used in SPE include silica-based (non-polar or medium polar analytes) <sup>53-55</sup>, graphitic carbon <sup>56</sup> (hydrophobic to hydrophilic analytes) <sup>57</sup>, hexagonal boron nitride (same property but better performance as graphitic carbon) <sup>58</sup>, ion-exchange based sorbents (negatively and positively-charged analytes) <sup>59</sup>, molecularly-imprinted polymers (MIPs) (complex matrices) <sup>60</sup>. Peptides present after tryptic digestion and SPE were analysed by ESI-MS and MALDI-MS.

Offline sampling formats in SPE had evolved into automated SPE systems coupled on-line<sup>61-64</sup> and in-line<sup>65-67</sup> for faster and more reproducible protein and peptide analysis in ESI-MS. One of the most recent developments in SPE is the utilization of microfluidic and on-chip analytical systems<sup>68</sup>. The most valuable characteristics of these systems are shorter analysis time and much lower sample consumption. Lastly, comparisons of lone or combined desalting strategies and sorbent brands were also highlighted in some researches<sup>69-71</sup>.

#### 1.4.1.2. Membrane-based desalting methods

Sample clean-up using membrane is based on separation of the sample (donor) solution from the acceptor (strip) by a semi-permeable membrane and allowing either the analytes or salts to pass through the membrane from the donor to the acceptor. The driving forces involved in membrane separation processes can be concentration difference that leads to molecular flux or transport of molecules, electric potential difference that leads to an electrical flux or transport of charge, and pressure difference (includes centrifugal devices) that leads to a volume flux or transport of bulk liquid or gas. With that, the mechanism of desalting using membranes can be in the form of dialysis, filtration, and electro-filtration/dialysis. Electric-field driven membrane-based desalting methods will be explained further in Section 1.5.

Dialysis is the technique when small molecules and salts are permitted to pass through a semi-permeable membrane that separates two-chambers. One chamber contains the acceptor liquid, while the other serves as the sample reservoir. Membranes used for dialysis with a particular molecular weight cut off (MWCO) separates the molecules based on their hydrodynamic dimensions. One of the most popular approaches of dialysis in desalting peptide samples prior to ESI-MS is on-line microdialysis with direct infusion to MS<sup>72, 73</sup>. Microdialysis, in combination with liquid chromatography, made the kinetic profiling of  $\leq 650$  Da biomarkers in a complex biological cell relatively fast. Microdialysis membranes are discriminating towards larger peptides or proteins, thus offer elegant solutions for the



separation of molecules with significant molecular size difference. However, major concerns limit the applicability of microdialysis<sup>74-76</sup>. For example, because dialysis is concentration gradient-driven, the acceptor liquid must be constantly replenished and this causes excessive dilution of the dialysate. Secondly, dialysis is not compatible with high throughput sample preparation because a dialysis process requires relatively longer sample cycle time. Thirdly, the amount of analyte recovered depletes over time as the analytes is exhausted in the donor channel.

In some cases wherein dialysis is not the suitable technique for sample cleanup for biological samples prior to ESI-MS, sample filtration or centrifugation can be employed to isolate liquid from solid particles. This method is typically in conjunction with peptide/protein precipitation and thus, requires relatively longer process time. Moreover, centrifugation is difficult to automate and the acquisition of the supernatant is critical. Incomplete protein or inorganic salt precipitation shortens column lifetime.

#### **1.4.2. SDS removal**

##### **1.4.2.1. SPE-based SDS removal methods**

Ion-exchange columns have been used to remove SDS from protein samples<sup>77</sup>. Columns used in ion-exchange can be anionic<sup>78</sup> or cationic<sup>79</sup>. For anion-exchange, positively-charged exchange resin is used. The SDS present in the loading buffer, e.g. trifluoroacetic acid in water, is adsorbed on the anionic-exchange column, while the peptides are eluted with a gradient of acetonitrile. SDS is then desorbed from the column when the high level of TFA-containing acetonitrile mobile phase is reached. For cationic-exchanger, the peptides are adsorbed on the ion-exchanger, and then desorbed prior to analysis. Various functional groups have been used in the ion-exchange resins, of varying strength as anion or cation-exchangers. In recent times, tandem configuration of this system with a LC column has been applied.

#### 1.4.2.2. Membrane-based SDS removal methods

The most prominent membrane-based SDS removal method is filter-aided sample preparation (FASP). The inception of the FASP method occurred when a commercial microcentrifuge filtration device (spin-filter) was employed for the removal of contaminants from protein samples solution prior to in-solution digestion<sup>80</sup>. The sample, diluted in buffer, was placed in the upper chamber of the filtration device and was centrifuged at 4500 x g. The proteins in the filter were then washed with water and buffer and the solution was centrifuged again. The filtrate was discarded and the protein was reconstituted with the buffer before reduction, alkylation, and digestion. A highly acidic bovine serum albumin (BSA) sample prepared in highly saline solution and detergent containing 2% SDS was used as a representative solution for a typical protein sample. Quantitative analysis for the degree of removal of SDS was not performed, however a high recovery of BSA peptides indicated an effective removal of SDS. It also showed an efficient tryptic digestion as a result of the removal of SDS and salts. The filtration device acted as reactor for the removal of SDS, buffer exchange, solubilization, and protein digestion in the upper chamber. Purified and digested peptides were recovered by centrifugation through the membrane. Sequential centrifugation of a protein sample in high concentration of urea to remove SDS was demonstrated using a spin-filter unit<sup>81</sup>. Urea can increase the critical micelle concentration of SDS, so that the protein is dissociated from the SDS and is then separated by the centrifugal filters. FASP

Employing FASP as an SDS depletion technique has been compared to other commonly used sample preparation protocols in proteome workflows. Comparison between FASP-gas phase fractionation (GSF) and SDS-PAGE sample preparation techniques was performed<sup>82</sup>. The number of identified proteins and peptides after LC-MS/MS analysis obtained using each approach was compared. FASP-GSF was determined to be more efficient in sample clean-up compared to SDS-PAGE. Xia and co-workers<sup>83</sup> compared FASP and

integrated on-line hollow fiber membrane interface for SDS removal, followed by an immobilized enzyme reactor for protein digestion. The use of hollow-fiber membrane proved to be more efficient and faster than FASP. Tanca et al.<sup>84</sup> compared the performance of direct tissue trypsinization, in solution digestion, and FASP as sample preparation techniques for formalin-fixed paraffin-embedded (FFPE) liver tissue. The methods employed for SDS depletion in the latter two workflows were spin column and molecular-weight cut-off centrifugal filtration, respectively. Direct tissue trypsinization does not use SDS or detergents in its process. The peptide mixtures obtained from the three techniques were analyzed with LC/MS and data were evaluated in terms of reproducibility and differential distribution of protein/peptide identifications according to subcellular localization. Direct tissue trypsinization resulted in lower reproducibility but high-MW proteins were preserved. In-solution digestion and FASP lost high-molecular weight protein but produced good results for hydrophobic and membrane proteins. Based on their study, FASP identified more proteins/peptides and ISD yielded higher reproducibility. Kachuk and co-workers<sup>85</sup> assessed and compared eight SDS depletion methods, namely in-gel digestion, protein precipitation by acetone or with TCA, detergent precipitation with KCl, strong cation-exchange, sample purification with Pierce detergent removal cartridges, and FASP. Among all of the mentioned methods, FASP produced the highest percentage of SDS removed but also showed significant sample loss of about 60%. Precipitation of protein with acetone resulted in an increase in protein and peptide recoveries of 17% and 40%, respectively, making it the most favored strategy for SDS removal in proteome analysis using LC-MS.

Some disadvantages have been pointed out when using FASP for sample clean-up. First, concerns arose when production of originally used Millipore Microcon centrifugal filters was discontinued<sup>86</sup>. A comparative study between various centrifugal filters was performed. Second, sample loss is also a major concern when using FASP. Erde and co-workers<sup>87</sup>

developed a method called enhanced FASP (eFASP) to circumvent this issue. Urea was substituted with 0.2% deoxycholic acid during digestion. The efficiency of tryptic digestion increased and cleanup steps for deoxycholate sodium salts were avoided.

#### 1.4.2.3. Precipitation SDS removal methods

There are two main strategies for the separation and removal of SDS from protein in a sample solution through precipitation. Proteins can be precipitated out from the solution containing SDS by addition of reagents such as ammonium acetate in methanol<sup>88</sup> or cold acetone<sup>89</sup>. In the same way, SDS can be precipitated out from the sample solution using guanidine hydrochloride<sup>90</sup>, chloroform/methanol<sup>91</sup>, and binding material MIL-101<sup>92</sup>.

### 1.5. Electric-field driven sample clean-up

One of the major issues of membrane filtration is membrane-fouling, wherein the analytes are concentrated or deposited on the surface of the membrane or in the membrane pores. This phenomenon decreases the performance of membranes and requires high-energy use, higher cleaning frequency, and causes shorter life span of the membrane. Membrane-fouling also causes noticeable reduction of analyte recovery, most especially in dead-end filtration where the feedstock is perpendicular to the membrane<sup>93, 94</sup>.

The application of an electric field, in conjunction with the use of membranes (also called electrodialysis) as a sample preparation step is employed to mitigate the issue caused by membrane-fouling. Typically, an electrodialysis system is composed of a donor channel and an acceptor channel, each containing an electrode, and coupled to a chromatographic or electrophoretic separation system. When an electric field is employed in a membrane-based process, the electric potential gradient drives the electromigration of charged molecules through the membrane towards the oppositely charged electrode. With this, the flow of analytes is caused by concentration gradient and electric potential<sup>74</sup>. More detailed theoretical description of electrodialysis is presented elsewhere<sup>95</sup>.

Electrodialysis is preferably applied only to charged species of greater mobility, whereas molecules with greater diffusivities can be removed in membraneless dialysis. In ordinary dialysis, back-diffusion occurs when the concentrations on both sides of the membranes are equal. When electric field is added as one of the driving forces of the separation process, the electric potential maintains the net transport of the charged analytes to the acceptor channel until the back-diffusion equals the electrical flux. In this manner, the analytes are almost quantitatively transported from the donor towards the acceptor channel. In addition, migration and diffusion of the analytes towards and through the interface is the rate-limiting step in a dialysis sample clean-up. The application of electric field hastens the sample preparation process.

The addition of an electric field in a membrane-based separation process has improved the performance of the sample preparation technique<sup>95, 96</sup> and electrodialysis has been applied online, coupled to chromatographic or electrophoretic separation systems<sup>97</sup>. The performance of the electrodialysis system is described in terms of an enrichment factor, which is (1) the concentration of the species in the acceptor phase after the sample preparation in relation to the original concentration in the sample. In the absence of an electric field, the concentration in the acceptor chamber can never be higher than in the donor chamber, thus the enrichment factor can never be greater than 1. In the presence of an electric field in a system with a moving donor phase, the enrichment factor for approaches to the maximum value. In practice, the applied electric potential can never be higher than 10 V to avoid thermal deterioration of standard cellulose membranes.

The enrichment factor can also be described based on the (2) sample flow-rate. An increase in the transported analyte molecules through the membranes leads to a higher enrichment factor per unit of time. The sample preparation process is performed over a shorter period of time, which translates to better sensitivity in an analytical method<sup>97</sup>. Aside

from the parameters mentioned that have direct effect on the enrichment factor in an electrodialysis system, one major practical consideration that influence an electrodialysis process is the composition of the sample. It was discovered that analyte recoveries decrease significantly with increasing sample ionic strength <sup>95, 97</sup>. The electric field strength in both channels decreases as conductivity is increased. Therefore, pure water is almost always used as the acceptor phase to maximize the electric field.

Dasgupta and co-workers <sup>98</sup> developed an in-line three-compartment electrodialytic salt remover for isotonic sample solutions containing cytochrome-C, myoglobin, and lysozyme as test proteins prior to ESI-MS analysis. The central compartment is confined by cation-exchange and anion-exchange membranes, where platinum electrodes are situated on both outer membranes. The test analytes have low electrophoretic mobilities compared to the inorganic ions, leading to the removal of the salts from the sample solution. The salt-free sample is directed to the ESI source for analysis. Good quality ESI-MS spectra were obtained after the inorganic ion removal.

Kachuk et al. <sup>99</sup> developed an automated electrophoretic device for SDS depletion from protein standards and an enriched membrane fraction from *E. coli*. prior to bottom-up MS/MS and LC-ESI-MS. As shown in Figure 1.5.1., the sample cartridge (1) consisted of four channels (2) and a molecular weight cut-off filter (4) positioned in each side of the sample cartridge sealed by custom gaskets (5). Protein solutions were introduced into the channels through the access ports (3) using a pipette. The cathode (6) and anode (7) chambers were filled with the electrolyte. All parts are secured between two aluminum plates (8). The device was run at a constant current of 40 mA for an hour with occasional mixing. The  $DS^-$  ions passed through membranes towards the positive electrode under the influence of the electric field, leaving behind the protein solution free of the surfactant. 99.9% removal of the surfactant and 90-100% intact protein recovery was obtained.

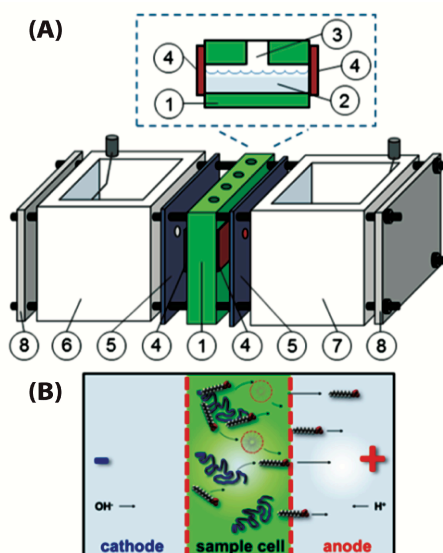


Figure 1.5.1. A. Model of SDS depletion apparatus. The sample cartridge (1) bordered by molecular weight cut-off filter (4). B. Applied electric field drives the anionic SDS across the membrane towards the anode, leaving the proteins in the sample cell <sup>99</sup> (with permission to use).

The applicability of electrodialysis is not only for a specific charged analyte, but for all charged compounds in the sample matrix. If the sample contains compounds of the same charge and molecular size as the analytes, there will be no gain in selectivity <sup>96, 97</sup>. Another practical limitation of electrodialysis is when dealing with samples containing high content of high-molecular-mass components, such as plasma. The electric field causes charged macromolecules to be irreversibly pulled against the membrane, thus reducing membrane permeability. Potential can be reversed after the sample preparation process is completed to remove charged compounds on the membrane surface, thereby reducing the possibility of membrane-fouling <sup>97, 100</sup>.

## 1.6. Electric-field and capillary electrophoresis

Fundamentally, electric field is used in capillary electrophoresis to separate components in a mixture. This separation of charged species or ions, which is carried out in a capillary containing a separation solution, occurs through the movement of these ions towards

an opposing charge (opposite attracts) when electric field is applied. For example, in the absence of an electric field, the motion of charged particles is in random. When the electric field is applied, charged species begin to move in a less random manner wherein cations (positively charged particles) move toward the cathode (negatively charged electrode) and anions (negatively charged particles) move toward the anode (positively charged electrode). Similarly charged species can also be separated based on the differences in the mass-to-charge ratio. The basic instrumental configuration for CE is composed of fused-silica capillary with an optical viewing window, a high voltage power supply, two electrode assemblies, two buffer reservoirs, and a detector (typically UV).

The separation of both large and small charged molecules by application of high voltages generates electrophoretic and electroosmotic flows for the charged analytes and the bulk solution, respectively. Electrophoretic mobility of the analytes is described by the following equation

$$\mu_{ep} = \frac{v_{ep}}{E} = \frac{L_d/t_m}{V/L_t} \quad (1.1)$$

where:

$\mu_{ep}$  = electrophoretic mobility

$v_{ep}$  = electrophoretic velocity

$E$  = electric field strength

$L_d$  = capillary length to the detector

$t_m$  = migration time

$V$  = voltage

$L_t$  = total length of the capillary

In electrophoresis, migration time is the time it takes for the analytes to migrate from the beginning of the capillary to the detector window. Equation 1 is only useful for determining



the apparent velocity, and not the actual mobility of the analytes. To determine the electrophoretic mobility, electroosmotic flow has to be accounted for. The electroosmotic flow is defined by the Smoluchowski<sup>101</sup> equation

$$v_{eo} = \frac{\epsilon\zeta}{4\pi\eta} E \quad (1.2)$$

where

$\epsilon$  = the dielectric constant

$\zeta$  = zeta potential measured at the plane shear close to the liquid-solid interface

$\eta$  = viscosity of the buffer

EOF exists in any electrophoretic systems and occurs whenever the liquid near a charged surface is placed in an electric field resulting in the bulk movement of fluid near that surface. EOF is controlled through capillary surface modifications<sup>102</sup>. As most of the commercially available capillaries have siloxane group on the surface, interaction of this surface with base (NaOH or KOH) causes hydrolysis of the siloxane group, thus creates a negatively-charged surface. The negatively-charged wall attracts cations that are hydrated from the electrolyte solution, creating an electrical double layer. Upon application of voltage, the cations in the diffused part of the double layer are attracted towards the cathode, carrying water with them. This results to the buffer solution net flow towards the negative electrode. The EOF has to be controlled or suppressed at times to run successful CE analysis and makes it possible to simultaneously analyse cations, anions, and neutral species in one single analysis. At neutral to high pH, the EOF is quite high than the electrophoretic migration and all species are dragged towards the negative electrode. The order of migration for the analytes to the detector would be cations, neutrals, and anions. To measure the EOF, neutral solute (methanol, acetone, mesityl oxide) can be injected and then migration time is measured.

Other factors affecting a successful capillary electrophoresis are capillary dimensions, voltage and temperature, properties (e.g. pH and viscosity) of the separation or buffer solution,

and temperature. Capillary diameters range from 20-200  $\mu\text{m}$ , and the smaller is the capillary, the better is the efficiency. However, too small capillaries result to poor limits of detection because of the very small path length and sample loadability. In addition, narrow capillaries are prone to clogging, thus filtering the buffers and sample solutions is imperative before loading. For electrokinetic injection (injection of charged analytes by voltage application), the total amount of ions injected into the capillary in electrokinetic injection after a specified time is directly proportional to the cross-sectional area of the capillary<sup>103</sup>.

The use of highest possible voltage during CE analysis results in the shortest separation time as the electroosmotic and the electrophoretic velocities are directly proportional to the field strength, as shown in equations 1.1 and 1.2. The limiting factor, however, for the amount of voltage is Joule heating, which is inherent in electrophoretic separations. Joule heating is the heat produced when an electrical current is passed through the buffer solution in the capillary. Consequently, temperature is strictly controlled during CE analysis as it is crucial to reproducible separations in CE. Capillaries with small inner diameter or longer length are used to control temperature. As the temperature gradient within the capillary is parabolic, as capillary inner diameter increases, the temperature gradient from the center of the capillary and the wall also increases. Current also increases as the diameter increases<sup>104</sup>.

A simple way to determine the coping capability of a CE method with regards to the heat that results from applied voltage and buffer concentration is by creating an Ohm's Law plot. The resulting currents at different voltages are plotted in a graph of current versus voltage, and a linear relationship must be established. The point at which the line deviates from linearity indicates the maximum possible voltage to be used in the specific buffer system/capillary combination. Monitoring the current during CE runs is therefore imperative as heat dissipation capacity of the instrument may be exceeded. This is true as well when the

heat is sufficient to boil the buffer locally indicated by the presence of bubbles, and results to breaking of the electrical circuit.

### **1.7. Research aims**

The availability of various strategies for desalting and SDS removal as sample preparation techniques in proteomics has expanded the breadth of possibilities for protein analysis. Significant amount of research had been done through the years on SPE systems for the removal of salts and surfactants as a sample preparation step. Membrane-based desalting and surfactant removal sample preparation systems are also applied, most prominent currently is FASP. Notable incremental advances for both SPE and membrane-based systems are presented in the literature to circumvent the limitations of the fundamental techniques. Precipitation is also a practical method for the removal of surfactant and studies were mostly focused on the discovery of new precipitants for high analyte recovery.

The use of electric field in a membrane-based sample preparation system, more specifically in desalting and SDS removal from biological matrices, caught the curiosity and attention of research groups in recent years. As presented in the previous section, the increase in analyte recovery, improvement of sensitivity, and prevention of membrane-fouling that occurs in dialysis are attractive characteristics of electric-field driven sample clean-up strategy.

One of the main challenges, however, for electrodialysis is the intricacy of constructing the device. This issue limits the reproducibility of the method. As also pointed out in the previous section, membrane-based systems are prone to fouling, most especially for complex biological matrices with high-molecular mass components. A simple, low cost, less laborious, electric-driven sample preparation strategy, that is membraneless, would be a suitable alternative for desalting and surfactant removal.

In this work, capillary electrophoresis is being used to selectively remove inorganic anions and dodecyl sulfate from standard peptides and digested protein sample solutions. The idea of this research project came about when Quirino<sup>105</sup> selectively removed cationic species from small sample volumes under the influence of an electric field in capillary electrophoresis (CE). The analytes were prepared in a sample solution of very low salt content. The efficient removal of the cationic species was performed using a commercial Agilent CE instrument, without the use of membranes. The method was simple, low cost, and less laborious than mostly-used sample preparation techniques.

This thesis project aims to develop an electric-field driven sample preparation technique for the removal of salts and SDS that is membraneless, less laborious, simple, environment-friendly, without any complex construction of a cleanup device, and can be applied for  $\mu\text{L}$  sample volumes. This research describes the results of a systematic investigation of electric-field driven desalting and SDS removal techniques for high conductivity biological samples, as well as samples containing micelles, prior to the analysis of peptides and peptides resulting from tryptic digestion of proteins in ESI-MS. The sample preparation steps were performed in commercial Agilent CE and PrinCE instruments, and in an online device coupled to ESI-MS. It is necessary to note that separation of peptides by LC-MS is essential in bottom-up proteomics. For this work, however, all MS analysis are not integrated to an LC platform, rather, samples are introduced to the MS instrument by direct infusion.

In Chapter 2, a one-step electrokinetic desalting of a microliter-sample solution with high salt content and angiotensin I peptide before ESI-MS analysis will be presented. The method, performed in a CE instrument, successfully removed inorganic anions electrokinetically and retained analytes in the sample vial by utilizing an acidic extraction solution and applying electric field. The simple and membrane-less method was performed by

controlling the apparent electrophoretic mobilities of the inorganic anions and the analytes at a boundary located at tip of a fused-silica capillary and sample solution. The effect of the electroosmotic flow was minimized by the acidic extraction solution. The effect of the voltage applied, desalting time, concentrations of inorganic anions, and dimension of the capillary were investigated to optimize the removal of inorganic anions and the retention of the analytes. The method was also applied for the removal of chloride ions in a solution containing low concentrations of arsenic and vanadium prior to inductively-coupled plasma-MS.

In Chapter 3, a simple and fast electrokinetic removal method of dodecyl sulfate ( $\text{DS}^-$ ) from an SDS micellar solution of peptides and tryptic digested proteins will be proposed. The  $\text{DS}^-$  removal was performed in a CE instrument, using a fused-silica capillary and an acidic extraction solution that contains 40% organic solvent, acetonitrile (ACN). The ACN initiated the collapse of the micelles at the boundary between the organic solvent and the sample solution, releasing the peptides (i.e. micelle-to-solvent stacking or MSS in CE) that caused the separation of the peptides from the surfactant. The electric field caused the electrokinetic migration of the  $\text{DS}^-$  monomers into the capillary towards the positive electrode. The peptides were left inside the sample vial, and were directly infused into the ESI-MS for elucidation and identification. The concentration of the organic solvent and  $\text{DS}^-$  removal time were optimized for maximum efficiency and performance of the developed method. The  $\text{DS}^-$  removal strategy obtained higher peptide % recoveries when compared with precipitation by cold acetone.

In Chapter 4, a simple electrokinetic device for the removal of SDS coupled online with ESI-MS will be presented. Significant improvements of ESI-MS signals of bradykinin and peptides from tryptic digestion of bovine serum albumin (BSA) by the synergistic effects of electric-field driven MSS and  $\text{DS}^-$  removal was obtained. The device was composed of a pressure source, voltage source, and a collection of fused-silica capillaries of different

dimensions, herein referred as a multi-capillary assembly. The extraction capillary, which is the most essential segment of the multi-capillary assembly, was optimized in terms of length and internal diameter (i.d.). Experimental parameters, i.e. voltage applied, voltage application time, and simultaneous pressure and voltage application, were optimized for efficient and successful  $\text{DS}^-$  removal operation. Approximately  $3.5\mu\text{L}$  was required for processing of one sample, which is valuable in proteomic workflows, wherein only low sample volumes are usually available.

Chapter 5 presents conclusions and future directions.

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## Chapter 2

### **One-step selective electrokinetic removal of inorganic anions from small volumes and its application as sample clean-up for mass spectrometric techniques**

#### **2.1. Chapter Summary**

The presence of inorganic anions in a sample interferes with mass spectrometric (MS) analysis. Here, a simple method to remove these ions from a liquid sample in one-step is described. The inorganic anions present in a 50  $\mu\text{L}$  sample were extracted into a low pH solution inside a 200  $\mu\text{m}$  i.d. 33 cm long capillary by the use of an electric field. The selective removal of unwanted anions and retention of target analytes was accomplished by control of the apparent electrophoretic velocities of anions and analytes at a boundary that separated the sample and extraction solution. No physical barrier (e.g., membrane) was required and with the boundary situated at the tip of the capillary, efficient removal of inorganic anions (e.g., > 80% removal) and good recovery of target analytes (e.g., > 80% recovery) were achieved. The time required for removal of the inorganic anions was found to depend on their initial concentrations. The removal process was investigated using different concentrations of bromide and nitrate (as potassium salts) and negatively chargeable drugs as target analytes. This micro-sample clean-up technique used no organic solvents and little consumables and was studied to the determination of 0.6  $\mu\text{g/L}$  arsenic and 8.3  $\mu\text{g/L}$  vanadium in 500 mg/L sodium chloride using inductively coupled plasma MS and 50  $\mu\text{M}$  angiotensin I in 1000 mg/L sodium chloride using electrospray ionisation MS. Micro-sample clean-up was performed for 45 min at 3 kV in both demonstrations. The calculated recoveries for the metals at trace levels were 110-130%, and for the peptide was 103.8%.

## 2.2. Introduction

Mass spectrometric (MS) analysis of environmental, food, biological and other samples can often be problematic when inorganic ions are present in the samples <sup>1-4</sup>. In inductively coupled-plasma-MS (ICP-MS) with standard sample introduction into an argon (Ar) plasma, high chloride matrices can result in the formation of polyatomic molecular interferences, especially for arsenic  $^{75}\text{As}^{+1}$  (from  $^{40}\text{Ar}^{35}\text{Cl}^{+}$ ) and vanadium  $^{51}\text{V}^{+1}$  (from  $^{35}\text{Cl}^{16}\text{O}^{+}$ ). In low resolution (high sensitivity) mode ICP-MS, chloride levels near 1 g/L may lead to signals equivalent to 0.5 µg/L of arsenic and 26 µg/L of vanadium. Thus, the presence of chloride prevents the reliable quantitation of these metals at the µg/L levels. In ESI-MS, the presence of high concentrations of inorganic salts causes a loss of ionization efficiency and this is commonly known as matrix effects that cause signal suppression or enhancement <sup>5</sup>. The accumulation of salts over continuous use of equipment also negatively affects the performance of ESI-MS instruments <sup>6,7</sup>.

For both ICP-MS and ESI-MS, the most commonly applied sample preparation methods for removal of inorganic ions (desalting) have been solid-phase extraction <sup>8,9</sup>, liquid-liquid extraction <sup>10,11</sup>, electrophoresis <sup>12-14</sup>, chromatography <sup>15-18</sup> and dialysis <sup>19,20</sup>. These methods are typically labour-intensive, time consuming, and can result in loss of analytes when applied to small volume samples. In ICP-MS, spectral interferences can be minimized using the high resolution mode, but results in an increase in the limit of detection. The addition of interference reduction technologies in ICP-MS instrumentation, such as collision and reaction cells, has also been developed to solve this issue <sup>21-24</sup>.

In ESI-MS, innovative microscale devices mainly integrated online have been reported. For example, Dasgupta and co-workers developed a three-compartment flow-through device that allowed the salt ions to be removed from the central channel through two ion-exchange membranes (anion and cation) under the influence of an electric field <sup>25</sup>.

Federov and co-workers developed a microfabricated device that utilized the difference in diffusivities of ions and analytes through a high counter-flow rate of sample solution and salt-free buffer, which were separated with an alumina membrane<sup>26</sup>. More recently, Zhang and co-workers reported membrane ESI (capable of reducing matrix effects from salts) that was inspired from paper spray ionization<sup>27</sup>.

The selective electrokinetic removal of cationic species from small sample volumes under field-enhanced conditions<sup>28</sup> in capillary electrophoresis (CE) has been reported previously<sup>29</sup>. The efficient removal of cationic species was obtained in samples with very low salt content. In the present study, a simple electrokinetic micro-sample clean-up technique to selectively remove inorganic anions from liquid samples with high salt content is described. This new membrane-free technique uses an acidic extraction solution (ES) inside a 200  $\mu\text{m}$  internal diameter (i.d.) capillary. Both ends of the 33 cm long capillary were dipped into a 50  $\mu\text{L}$  sample and ES. An electric field was used to selectively separate the inorganic anions from the target ionizable analytes in the sample by transfer of the inorganic anions into the ES inside the capillary. The separation was accomplished by control of the apparent electrophoretic velocities of analytes and inorganic anions at the tip of the capillary which was in contact with the sample. Significant removal of inorganic anions and high recovery of the target analyte species which remained in the sample solution were achieved. This environmentally friendly analytical sample preparation technique was demonstrated for the analysis of arsenic and vanadium by ICP-MS, and also for a peptide (angiotensin I) by ESI-MS in the presence of high concentrations of sodium chloride (NaCl) in the sample. Inorganic cations such as sodium ions, however, are retained in the sample and formed adducts with the analytes in ESI-MS. The adduction of alkali metal ions to analytes is a common occurrence in ESI-MS<sup>30,31</sup>. The adducts formed often have higher sensitivities than the protonated or deprotonated molecules. The adducts can be used for quantitation<sup>32,33</sup>.



Algorithms have been developed to accurately determine the molecule represented by molecular ion adducts in positive and negative mode in ESI conditions <sup>34</sup>.

## **2.3. Experimental Section**

### **2.3.1. Reagents and Stock Solutions.**

Acetic acid (>99.8%), phosphoric acid (85 wt.%), acetonitrile (ACN, HPLC grade), sodium dodecyl sulfate (SDS, >99.0%), sodium hydroxide (NaOH, >95%), sodium chromate ( $\text{Na}_2\text{CrO}_4$ , 98%), hexadimethrine bromide (HDMB, >94%), potassium bromide (KBr, >99%), potassium nitrate ( $\text{KNO}_3$ , >99%), NaCl (>99.5%), triethanolamine (TEA, >99.0%), methanol (MeOH, HPLC grade), and angiotensin I human acetate salt hydrate ( $\geq 90.0\%$ ) were obtained from Sigma-Aldrich (St. Louis, MA). Rosuvastatin (pKa 4.0, MW 481 g/mol), furosemide (pKa 3.8, MW 330 g/mol), indapamide (pKa 8.8, MW 365 g/mol) acidic drugs (>99%) were purchased from Sigma-Aldrich or Fluka (Buchs, Switzerland). Purified water was from a Milli-Q system (Millipore, Bedford, MA, USA). Stock solutions of the inorganic anions were 10 g/L of KBr,  $\text{KNO}_3$ , and NaCl in purified water. Stock solutions of the target compounds were 1 mg/mL of each drug and 100  $\mu\text{M}$  angiotensin I in purified water. Arsenic and vanadium atomic absorption standard solutions (1000 mg/L) were obtained from Sigma-Aldrich. ES was 5 M acetic acid at pH 2.5. All solutions were filtered through 0.45- $\mu\text{m}$  filters.

### **2.3.2. General CE procedure.**

CE was performed using an Agilent Technologies 3D-CE instrument (Waldbronn, Germany). Separation capillary was fused-silica from Polymicro (Phoenix, AZ) with 50  $\mu\text{m}$ i.d. and 360  $\mu\text{m}$ o.d.. New capillaries were flushed at 1 bar with 0.2 M NaOH (15 min), purified water (5 min) and then separation solution (10 min) prior to use. Sample injection was at 25 mbar for 6s.

Acids such as HCl, HNO<sub>3</sub>, and acetic acid were dismissed as extraction solution given that chloride and nitrate ions were used in this study. Different concentrations of acetic acid were studied ranging from 0.5 to 5 M acetic acid. 5 M acetic acid delivered the highest removal of inorganic anions when used as extraction solution at a given condition, and thus was used as extraction solution in this work.

### **2.3.3. *Capillary zone electrophoresis (CZE) with direct UV detection of Br<sup>-</sup>, NO<sub>3</sub><sup>-</sup>.***

Capillary (50 cm total) was 41.5 cm to the UV absorbance detector. The capillary was flushed at 1 bar with purified water (1 min) then the separation solution (4 min) prior to each run. The separation solution was 50 mM sodium phosphate at pH 2.5. Separation voltage and detection was at -20 kV and 200 nm, respectively. The method was linear from 5 to 100 mg/L for both anions in purified water with  $r^2 \geq 0.999$ . The %RSD (n = 5) of corrected peak area (peak area/migration time) using a sample with 50 mg/L each of KBr and KNO<sub>3</sub> was 3.5%. The samples were diluted with purified water to a target concentration of 50 mg/L of each salt based on the salt concentrations before removal. The sample after removal was diluted appropriately as needed.

### **2.3.4. *Capillary zone electrophoresis (CZE) with indirect UV detection of Cl<sup>-</sup>.***

Capillary was 24.5 cm (33 cm total) to the detector. A new capillary was flushed at 1 bar with 0.1% HDMB (10 min), purified water (1 min), then separation solution (5 min). The capillary was flushed at 1 bar with purified water (1 min), 0.1% HDMB (2 min), purified water (1 min), then separation solution (3 min). The separation solution was 20 mM Na<sub>2</sub>CrO<sub>4</sub><sup>2-</sup> and 20 mM TEA in 20% ACN. Separation voltage and indirect detection was at -10 kV and 254 nm, respectively. The method was linear from 5 to 1000 mg/L NaCl in purified water with  $r^2 \geq 0.999$ . The %RSD (n = 5) of corrected peak area using a sample with 50 mg/L NaCl was 1.1%. The samples were diluted with purified water to a target concentration of 50 mg/L

NaCl based on the NaCl concentration before removal. The sample after removal was diluted appropriately as needed.

### **2.3.5. *Micellar electrokinetic chromatography (MEKC) with UV detection of drugs.***

Capillary (50 cm total) was 41.5 cm to the detector. The capillary was flushed at 1 bar with purified water (1 min) then separation solution (4 min). The separation solution was 100 mM SDS and 10 mM H<sub>3</sub>PO<sub>4</sub> in 20% ACN. Separation voltage and detection was at -20 kV and 200 nm, respectively. The method was linear from 2 to 20 mg/L for all drugs in purified water with  $r^2 \geq 0.998$ . The %RSDs (n = 5) of corrected peak area using a sample with 10 mg/L of each drug was from 1.5 to 2.5%. The samples before and after removal were directly injected into the capillary.

### **2.3.6. *Direct infusion ESI-MS of angiotensin I.***

The ESI-MS experiment was conducted on an Agilent 6320 Ion Trap MS system with an Agilent G1607A ESI interface. Sample solution containing 50  $\mu$ M angiotensin I human acetate salt hydrate in 1000 mg/L NaCl was used. This concentration of NaCl showed dominant NaCl clusters in ESI-MS and caused suppression of the analyte signals.

The sample was flowed to the ESI-MS with an Agilent CE system using a 75  $\mu$ m i.d. fused silica capillary (1 m long) at 1 bar. ESI voltage was -4 kV. Spray chamber parameters were as follows: nebulizer pressure 15.0 psi, dry gas flowrate 5.0 L/min, drying gas temperature 325°C. Total ion scan was performed at 100 – 1200 m/z.

### **2.3.7. *ICP-MS of Ar and V.***

ICP-MS analysis was with an Element 2 Sector field ICP-MS from ThermoFischer (Waltham, MA). The sample solution was composed of 0.6  $\mu$ g/L arsenic and 8.30  $\mu$ g/L vanadium for ICP-MS analysis in 500 mg/L NaCl. This concentration of NaCl mimics the salinity of freshwater or inland water, where concentration of heavy metals such as arsenic and vanadium are closely monitored.

The analytes of interest ( $^{51}\text{V}$  and  $^{75}\text{As}$  isotopes) were measured using low ( $m/\Delta m = 400$ ) and high ( $m/\Delta m > 9500$ ) resolutions. 10 mg/L of  $^{115}\text{In}$  was used as internal standard. Operating parameters were as follows: plasma power 1350 W, cooling gas flowrate 15 L/min, auxiliary gas flow rate 0.8 L/min, sample gas 0.97 L/min optimized daily. The %RSDs ( $n=3$ ) for each determinations were  $< 3\%$ .

### 2.3.8. General procedure for removal of inorganic anions.

The removal of inorganic anions was performed using a commercial CE instrument from Agilent Technologies (3D-CE) (Waldbronn, Germany). The CE instrument, which was readily available in our laboratory, was used to conveniently establish the fundamentals of the method. The extraction capillary was fused-silica from Polymicro (Phoenix, AZ) with 360  $\mu\text{m}$  o.d.. A wide diameter capillary 200  $\mu\text{m}$  i.d. was used since the amount of ions electrokinetically extracted into the capillary was proportional to the capillary i.d.. According to Chien <sup>35</sup>, the total amount of ions  $i$  injected into the capillary in electrokinetic injection after a specified time ( $t$ ) is directly proportional to the cross-sectional area  $A$  of the capillary as shown in the following equation:

$$N_i = \int_0^t AC_{is}(v_{ei}t + v_b(t))dt \quad (2.1)$$

Where:

$N_i$  = total amount of ion species

$C_{is}$  = concentration of ion species  $i$  in the sample reservoir

$v_{ei}$  = electrophoretic mobility of the ion  $i$

$v_b$  = electroosmotic velocity

New capillaries were flushed at 1 bar with 0.2 M NaOH for 5 min, purified water for 2 min, and ES for 4 min. Pre-conditioning of the capillary before each sample treatment was carried out by flushing at 1 bar with 0.2 M NaOH for 0.5 min, purified water for 0.25 min, and ES for 0.25 min. A 50  $\mu\text{L}$  sample solution was placed in the cathodic vial while the ES

was placed in the anodic vial. Voltage at 3kV with the cathode at the sample vial was applied while maintaining the capillary temperature at 20°C. Higher voltages led to voltage drops and excessive bubble formation at the sample vial, especially for high salt content samples. At the end of each working day, the electrodes were washed and stored in purified water to remove salt residues.

## 2.4. Results and Discussion

### 2.4.1. *Selective electrokinetic removal of inorganic anions through a purely aqueous boundary*

Figure 2.4.1.1 shows the mechanism for the selective removal of inorganic anions from a small volume sample solution (i.e., 50  $\mu$ L), with the analytes being retained in the sample. (A) is the starting situation. A 200  $\mu$ m capillary (33 cm) with acidic ES was dipped into the sample. The other end of the capillary was dipped in a vial with the ES. A purely aqueous boundary between the sample solution and the ES was established at the tip of the capillary that was in contact with the sample. A voltage with the cathode at the sample solution was applied as shown in (B). The resultant  $v_{eof}$  that was directed towards the sample solution was slow because of the low pH of the ES. The negatively and positively charged species in the sample migrated to the anode and cathode, respectively.

In (B), the apparent velocities ( $v_{app}$ ) of inorganic anions was towards the capillary, resulting in removal of inorganic anions from the sample, whereas  $v_{app}$  for the analytes was towards the sample solution, leading to retention of analytes in the sample. The  $v_{app}$  was the sum of the electrophoretic velocity of the compound ( $v_a$ ) and velocity of the EOF ( $v_{eof}$ ), with  $v_a$  being equal to the product of electrophoretic mobility ( $\mu$ ) and electric field strength ( $E = \text{applied voltage/length of the capillary}$ ). In the case of inorganic anions,  $v_a > v_{eof}$  owing to the acidic ES, thus the  $v_{app}$  was directed towards the capillary. These small anions have very high

$\mu$  values and their mobilities were unaffected by the low pH of the ES. These anions were therefore extracted into the capillary when the voltage was applied. This was measured in terms of their degree of removal in this study.

For neutral, cationic or weakly ionisable anionic components in the sample, the  $v_a$  was either equal to 0 or was in the same direction as the  $v_{eof}$ , thus the  $v_{app}$  was directed to the sample vial. These compounds were not extracted into the capillary and therefore remained inside the sample solution. This behaviour was measured in terms of recovery in this study. For weakly acidic analytes, the  $v_a$  was slow because even if these analytes were ionized in the sample solution, on reaching the boundary with the ES their ionization was suppressed due to the low pH of the ES. For cationic analyte, the  $v_a$  was directed away from the ES. The behaviour of amphoteric compounds depends on the sample pH, but even when these species were anionic and their migration was initially towards the ES, their charge was reversed by the low pH of the ES and their resultant  $v_{app}$  was away from the ES, leading to their retention in the sample.

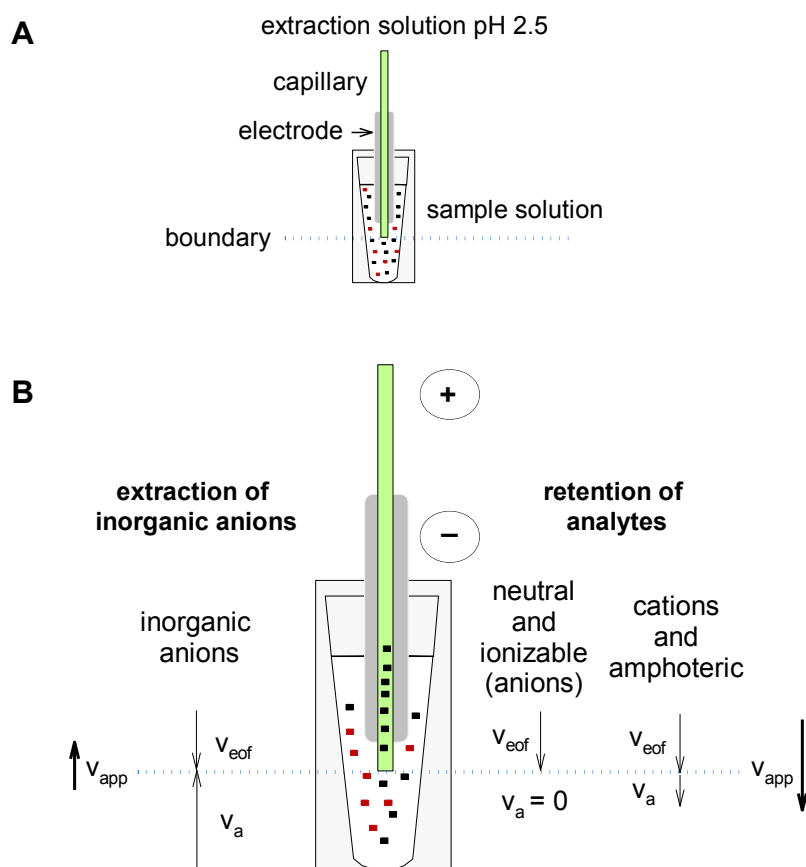


Figure 2.4.1.1. Mechanism of selective electrokinetic removal of inorganic anions from small sample volumes through a purely aqueous boundary. The figure shown in (A) was the initial condition. The sample solution contained the inorganic anions (black dots) and target analytes (red dots). A fused-silica capillary was filled with low pH extraction solution. The ends of the capillary were dipped into the sample vial (cathode) and a vial filled (anode) with extraction solution. (B) A voltage was applied at negative polarity. The inorganic anions were selectively removed from the sample solution because their apparent velocities ( $v_{app}$ ) were directed toward the capillary. The target neutral, cationic, and ionisable analytes remained in the sample solution because their  $v_{app}$  were directed toward the sample vial.

#### 2.4.2. *Proof of concept - removal of inorganic anions and recovery of negatively ionizable analytes*

The concept of selective electrokinetic removal of inorganic anions was verified using a mixture of inorganic anions and negatively ionisable analytes in a 50 mL sample solution.

The sample was 500 mg/L each of bromide and nitrate as potassium salts and 10 mg/L each of target analytes (i.e., rosuvastatin, furosemide, and indapamide) in purified water. The structures of the target drugs are given in Figure 2.4.2.1.

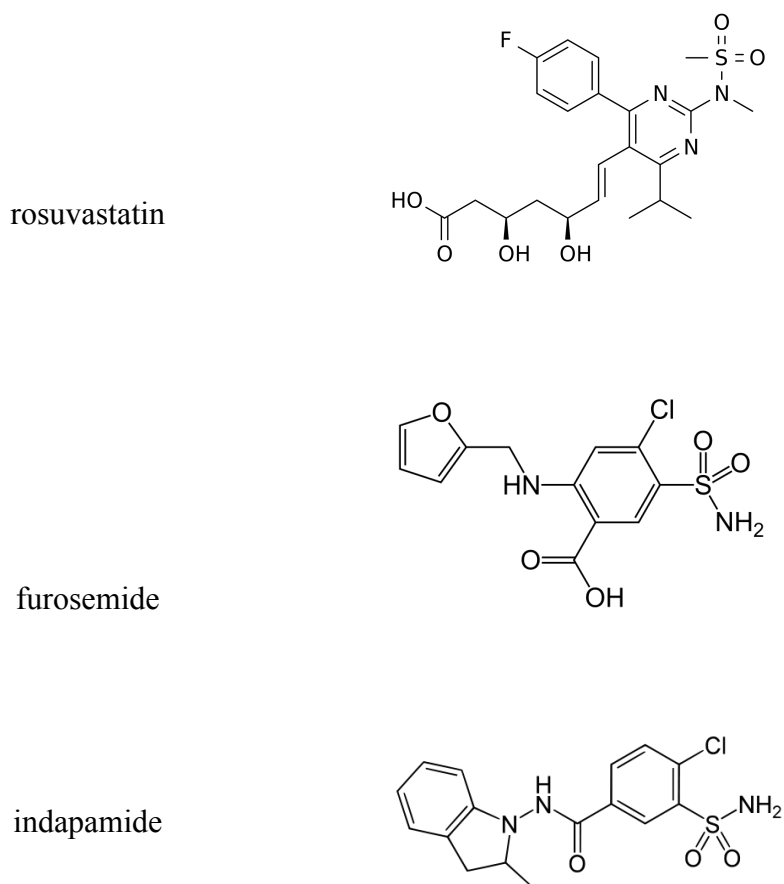


Figure 2.4.2.1. Structures of the negatively ionizable drugs tested

The negatively ionizable drugs were chosen as targets because their initial  $v_{app}$  was towards the ES under the conditions described in Figure 2.4.2.2 and therefore there is potential for their loss during the removal of inorganic anions from the sample. Figure 2.4.2.2 depicts the removal of bromide and nitrate (left y-axis) and the recovery of the target drugs (right y-axis). Removal was performed for 10 – 80 min with an applied potential of 91 V/cm across the extraction capillary. The percentage removal was calculated by dividing the concentration of the anions found in sample after removal by the concentration in the original sample ( $\times 100\%$ ). Analysis was performed by CZE with direct UV detection. Percentage



recovery was calculated by dividing the concentration of the drugs found in the sample after treatment by the concentration in the original sample ( $\times 100\%$ ). Analysis was performed by MEKC with UV detection.

A study of i.d. 25, 50, 100, 150, 200  $\mu\text{m}$  capillaries was performed. The biggest i.d. (200  $\mu\text{m}$ ) showed the highest percentage removal of inorganic ions at a given time. The shortest possible total length of 33 cm for accommodation of the capillary into the capillary cartridge was employed.

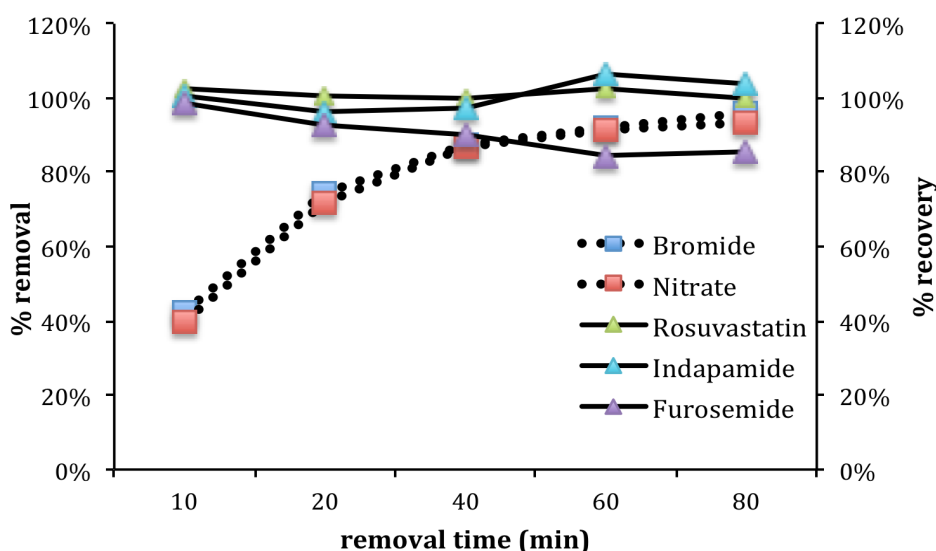


Figure 2.4.2.2. Percentage removal of  $\text{Br}^-$  and  $\text{NO}_3^-$  (500 mg/L each as K salts) and percentage recovery of negatively chargeable rosuvastatin, furosemide, and indapamide (10 mg/L each) with extraction times of 10-80 min. Removal conditions: 3 kV across a 33 cm length fused-silica capillary (200  $\mu\text{m}$  i.d.) filled with 5 M acetic acid as extraction solution. Inorganic anions (line dots) and negatively chargeable drugs (line solid).

The percentage removal increased from 10 to 40 min., with values of 88% and 87% being attained for  $\text{Br}^-$  and  $\text{NO}_3^-$ , respectively at 40 min. A further gradual increase in percentage removal was observed from 40 to 80 min since most of the anions had been

removed within 40 min. These results demonstrated that the target analytes could be retained in the sample while the inorganic anions were removed. The %removal for the salt samples was not as dramatic after 80% removal was achieved. The %recovery values for the drugs were typically > 80% when the %removal was  $\leq 80\%$ . The %recovery did not fall below 70% under all the conditions studied. The %recovery values were however < 80% for furosemide when the extraction times were longer. This indicated greater removal of furosemide compared to the other drugs tested. This was when there had been significant removal of the anions. Furosemide might have undergone decomposition after 60 minute removal time.

Representative electropherograms of the inorganic anions and the target analytes before and after 40 min inorganic ion removal are presented in Figure 2.4.2.3

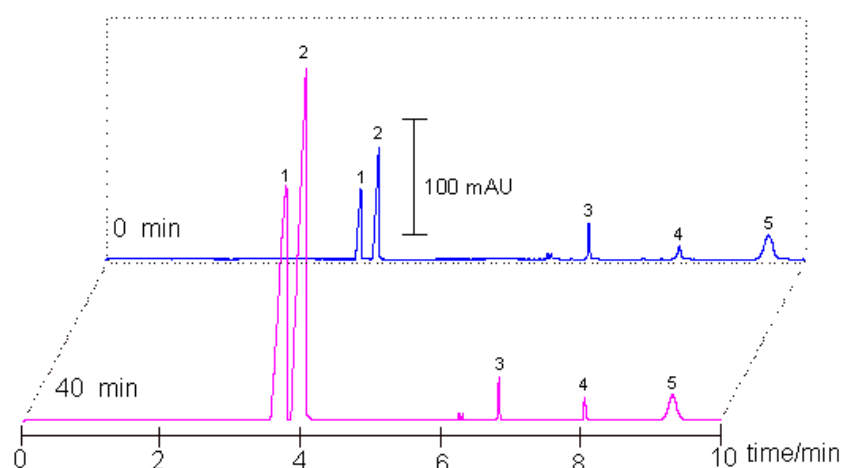
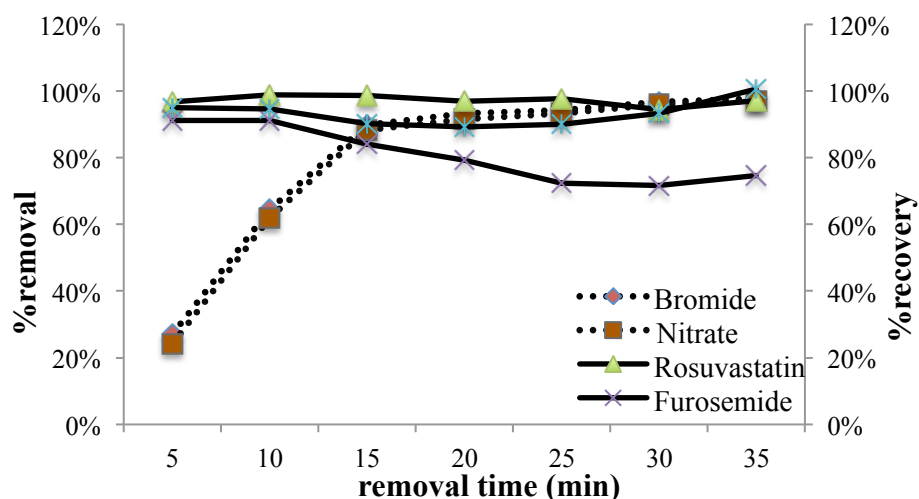


Figure 2.4.2.3. Representative electropherograms of inorganic salts  $\text{Br}^-$  (1),  $\text{NO}_3^-$  (2), and target analytes rosuvastatin (3), furosemide (4), and indapamide (5) before and after 40 min inorganic ion removal. Removal conditions: 3 kV across a 33 cm length fused-silica capillary (200  $\mu\text{m}$  i.d.) filled with 5 M acetic acid as extraction solution.

The same experiments were performed with 100 and 250 mg/L each of  $\text{Br}^-$  and  $\text{NO}_3^-$  as potassium salts in the sample. The results are shown in Figure 2.4.2.4 for the 100 (A) and 250 (B) mg/L salt sample, respectively.

**A**



**B**

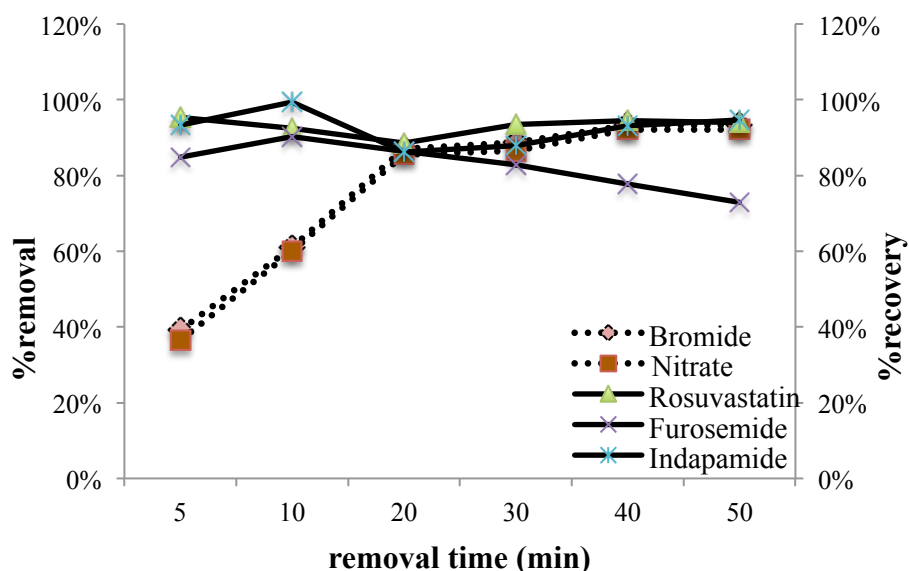


Figure 2.4.2.4. %removal of  $\text{Br}^-$  and  $\text{NO}_3^-$  of (A) 100 mg/Leach as K salts and (B) 250 mg/Leach as K salts), and %recovery of negatively ionizable drugs rosuvastatin, furosemide, and indapamide (10 mg/L each) with removal time from 5 to 35 min. Removal conditions: 3 kV across a 33 cm length fused-silica capillary (200  $\mu\text{m}$  i.d.) filled with 5 M acetic acid as extraction solution. Inorganic anions (line dots) and drugs (line solid).

The removal time was from 5 to 35 min and 5 to 50 min, correspondingly. The concentrations of the drugs were the same as in Figure 2.4.2.2. For the sample containing 100 mg/L salts, a percentage removal of  $>80\%$  was achieved for an extraction period of 15 min, whereas to obtain a similar percentage removal of inorganic anions from the sample

containing 250 mg/mL salts a longer removal time of 20 min was required. It was also noted that to attain a percentage removal of >80% for a 500 mg/mL salt sample, an extraction time of at least 40 min. was required. In summary, these studies showed that the time required to obtain significant removal of the inorganic anions was dependent on the initial concentration of inorganic anions in the sample.

The rate of salt removal decreased when the percentage removal of the anions reached >80%. This was attributed to the formation of hydroxide ions from electrolysis at the cathode, which were then transferred into the extraction capillary in competition with the salt ions in the sample. The presence of electrolysis was verified by high pH (>10) observed for samples that had been subjected to extraction for periods of  $\geq 15$  min.

#### **2.4.3. *Application for sample clean-up prior to ESI-MS***

The proposed sample clean-up approach for removal of inorganic anions prior to ESI-MS analysis was studied using a peptide as target analyte. The sample solution used contained 1000 mg/L NaCl and 50  $\mu$ M angiotensin I human acetate salt hydrate (MW = 1296.5). The sample was directly infused into the ESI-MS before and after sample clean-up, with the resultant ESI-MS spectra being displayed in Figure 2.4.3.1.

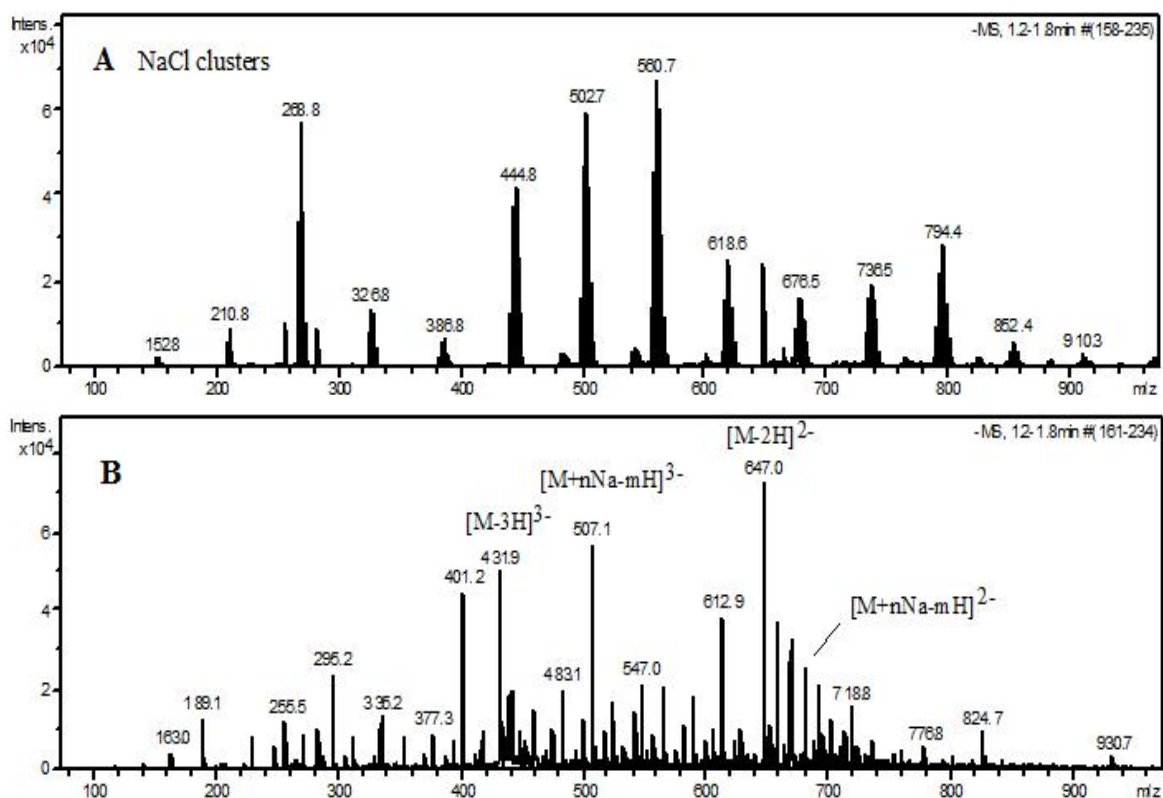


Figure 2.4.3.1. Negative ion mode ESI-MS spectra of 50  $\mu$ M angiotensin I in 1000 mg/L NaCl solution before (A) and after removal (B). Removal conditions are the same as Figure 2.4.2.2.

Figure 2.4.3.1A (prior to clean-up) shows the presence of NaCl clusters (e.g.,  $m/z$  268.8, 444.8, 502.7, and 560.7) and strong suppression of the ionization of angiotensin I, such that no signals corresponding to the peptide were detected. Figure 2.4.3.1B (after clean-up at 3 kV for 45min.) shows the detection of angiotensin I as  $[M-3H]^{3-}$  and  $[M-2H]^{2-}$  ions at 431.9 and  $m/z$  647.0, respectively. Analysis of the treated sample by CZE with indirect UV detection showed 90% removal of chloride ions. Also in Figure 2.4.3.1B, sodium adducts of the peptide (i.e.,  $[M+nNa-mH]^{3-}$  and  $[M+nNa-mH]^{2-}$ ) were also visible, since adduct formation with sodium ions is a reaction with peptides and similar compounds in ESI-MS. The total signal of the formed adducts ( $m/z$  647, 431.8, 507.1, and 680) was calculated and recovery with respect to the standard solution was determined. 103% recovery of the peptide

was obtained. It is also noted that a much smaller signal for the NaCl clusters are found in the ESI-MS spectrum since 100% removal was not achieved.

#### 2.4.4. Application as sample clean-up prior to ICP-MS analysis

The determination of low-level arsenic and vanadium by low resolution ICP-MS is hindered by the presence of polyatomic interferences of the same molar mass.  $^{40}\text{Ar}^{35}\text{Cl}^+$  and  $^{35}\text{Cl}^{16}\text{O}^+$  species interfere with the signals of  $^{75}\text{As}^+$  and  $^{51}\text{V}^+$  isotopes. ICP-MS analysis of solutions with increasing amounts of NaCl displayed increasing abundance of  $^{40}\text{Ar}^{35}\text{Cl}^+$  and  $^{35}\text{Cl}^{16}\text{O}^+$ , as shown in Figure 2.4.4.1.

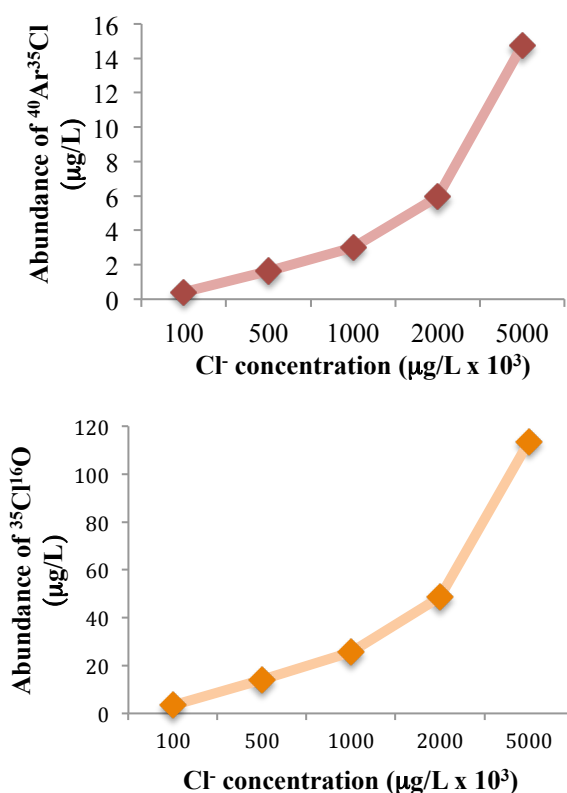


Figure 2.4.4.1. Corresponding abundances of  $^{40}\text{Ar}^{35}\text{Cl}$  and  $^{35}\text{Cl}^{16}\text{O}$  at increasing Cl<sup>-</sup> (as Na salt) concentration. ICP-MS analysis was done at low resolution.

The capacity of the proposed clean-up procedure to remove chloride from a sample solution in order to provide improved performance of low resolution ICP-MS of the above metal ions was therefore studied. The sample contained 0.60 μg/L arsenic and 8.30 μg/L

vanadium (based on quantitative high resolution ICP-MS which can remove chloride signal interferences) in a matrix of 500 mg/L NaCl. Low resolution ICP-MS of this sample provided results of 1.62  $\mu\text{g/L}$  arsenic and 16.30  $\mu\text{g/L}$  vanadium; that is, the arsenic and vanadium levels was significantly overestimated by 270% and 196%, respectively. Analysis of the same solutions by low resolution ICP-MS after clean-up at 3 V for 45 min gave values of 0.80  $\mu\text{g/L}$  As and 9.20  $\mu\text{g/L}$  V, which were 130% and 110% of the values obtained from high resolution ICP-MS, respectively.

## **2.5. Conclusions**

A simple, one-step selective removal of inorganic anions from small volume sample solutions was described. The mechanism of removal was based on the manipulation of the apparent electrophoretic velocities of inorganic anions and target analytes. The micro-sample clean-up technique is environmentally friendly since no organic solvents were involved and there was minimal use of consumables. Preparation of membranes was not required and the procedure was conducted with a 200  $\mu\text{m}$  i.d. capillary and a commercial CE instrument (or a home-built system consisting of a high-voltage power supply and suitable electrodes). Significant removal of  $\sim 90\%$  of inorganic anions within 45 min (at 3 kV) was achieved for samples containing 500 to 1000 mg/L of salts (up to 2.0 mS conductivity) and the improved detection of a peptide and important metals by MS techniques was demonstrated. Around 100% recovery of the peptide was obtained, which in part was due to the adduct formation of the peptide with sodium ions which is a reaction in ESI-MS analysis. The removal of anions from extremely saline samples was difficult because of the resultant high currents observed during the clean-up procedure. Dilution of very saline samples without compromise to detection is a possible solution. For example, a sample with 4.0 mS conductivity can be diluted two times with water before 45 min removal time.

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## Chapter 3

### **Electrokinetic removal of dodecyl sulfate micelles from digested protein samples prior to electrospray-ionization mass spectrometry**

#### **3.1. Chapter Summary**

In proteomics, dodecyl sulfate ( $\text{DS}^-$ ) as sodium salt is commonly used in protein solubilization prior to tryptic digestion, but the presence of the  $\text{DS}^-$  hampers the electrospray ionization mass spectrometric (ESI-MS) analysis. The development of  $\text{DS}^-$  depletion techniques is therefore important especially when dealing with small samples where there could be poor sensitivity due to sample loss or dilution during sample preparation. Here, we present a simple and fast electrokinetic removal method of  $\text{DS}^-$  from small volumes of peptide and digested protein samples prior to ESI-MS. The selective removal was accomplished using an acidic extraction solution (ES) containing acetonitrile (ACN) inside a fused-silica capillary that was dipped into the sample. The use of acidic ES suppressed the electroosmotic flow; allowing the electrokinetic movement of  $\text{DS}^-$  monomers and micelles into the capillary. The high amount of ACN present at the tip of the capillary served to collapse the micelles migrating into the capillary, thereby releasing the peptides that were bound to these micelles, facilitating peptide retention in the sample and efficient  $\text{DS}^-$  removal. Increased % MS signal intensity (SI) restoration of the peptide was observed, while  $\text{DS}^-$  removal was unaffected when the amount of ACN in the ES was increased. This is because of the micelle to solvent stacking mechanism (effective electrophoretic mobility reversal) working at high concentration of ACN for the improved recovery of the peptides. % MS SI restoration for the Z-Gly-Gly-Val and bradykinin peptides were 75-83% while % MS SI reduction of  $\text{DS}^-$  was up to 99% under optimal conditions, i.e. 40% ACN in the ES.

### 3.2. Introduction

Bottom-up proteomics is widely used to identify proteins and characterize their amino acid sequence. This approach uses a protease, i.e. trypsin, to digest protein mixtures to their peptide level of preferred mass range for electrospray ionization mass spectrometric (ESI-MS) analysis. Prior to digestion, denaturants, e.g. sodium dodecyl sulfate (SDS), are often used for the total solubilization and stabilization of protein complexes and hydrophobic protein solutions. Effective denaturation results from firm binding of the hydrocarbon tail of anionic dodecyl sulfate ( $\text{DS}^-$ ) to cationic proteins. Unfortunately, downstream analysis by ESI-MS is compromised by the presence of  $\text{DS}^-$  in the sample. Suppression of the signals of peptides occurs when SDS level is 0.01% (0.35 mM) or more, and  $\text{DS}^-$  clusters are immediately noticed the MS spectra<sup>1-3</sup>. Thus, efficient removal of  $\text{DS}^-$  is an integral aspect of proteomic workflows prior to ESI-MS. On the other hand,  $\text{Na}^+$  from SDS form adducts with peptides that can lead to increased ESI-MS sensitivity and these adducts aid in the identification of the target biomolecules. Thus, the removal of  $\text{Na}^+$  from SDS is not necessary<sup>4,5</sup>.

Sample preparation of biological mixtures can be laborious, most especially when dealing with small sample volumes. Numerous protocols, such as column-based<sup>6</sup>, membrane-based<sup>7</sup>, precipitation<sup>8</sup>, and solvent extraction<sup>9</sup>, have been made available to remove surfactant from samples. While these methods, especially filtration-based, can remove surfactants and salts<sup>10,11</sup>, there is the unpredictable loss of analytes, requirement for laborious and time-consuming procedures, and incomplete  $\text{DS}^-$  removal.

An electrophoretic microchip device was described for the effective removal of SDS in a standard peptide sample without micelles or salt and contained 25% MeOH<sup>12</sup>. We have reported a single-step and selective electrokinetic removal of cationic species<sup>13</sup> and inorganic anions<sup>14</sup> from  $\mu\text{L}$  sample volumes, demonstrating the simple use of an electric field and a capillary for microscale sample preparation. Here, we report the selective electrokinetic

removal of  $\text{DS}^-$  from a small volume micellar sample solution with salt. This simple microscale sample preparation technique was performed in a commercial capillary electrophoresis (CE) instrument and without any use of membranes or fabricated materials. The removal of  $\text{DS}^-$  and the retention of peptides were initiated by the synergistic effects of the electric field and the use of appropriate quantities of organic solvent in the extraction solution (ES). The developed platform was demonstrated for the analysis of small peptides (Z-Gly-Gly-Val (ZGGV) and bradykinin, and tryptic digested proteins (concanavalin A (conA), bovine serum albumin (BSA), and conalbumin (CA)) by ESI-MS.

### **3.3. Materials and Methods**

#### **3.3.1. Reagents and Stock Solutions.**

Sodium hydroxide (NaOH, >95%), hydrochloric acid (HCl), acetonitrile (ACN), methanol (MeOH), acetone, concentrated acetic acid (HAc), SDS, dihydroxybenzoic acid (DHBA), tris-HCl, 1,4-dithiothreitol, iodoacetamide (IAA), ammonium bicarbonate ( $\text{NH}_4\text{HCO}_3$ ), trypsin from bovine pancreas were from Sigma-Aldrich (St. Louis, MA) or Fluka (Buchs, Switzerland). Peptides ZGGV and bradykinin, and protein samples CA (76 kDa), conA (26.5 kDa), BSA (69 kDa) were from Sigma-Aldrich. Purified water was from Milli-Q system (Millipore, Bedford, MA, USA). Stock solutions of the peptides were 200  $\mu\text{M}$  in purified water. Stock solution of 500 mM HAc was prepared by appropriate dilution of concentrated HAc with purified water. The background electrolyte (BGE) for CE was prepared by dilution of the stock solution of HAc with purified water.

#### **3.3.2. CE procedure for bradykinin.**

Electrophoresis was performed using a CE instrument from Agilent (Waldbronn, Germany). Fused-silica capillary from Polymicro (Phoenix, AZ) with 360  $\mu\text{m}$  o.d. and 50  $\mu\text{m}$  i.d. was used. New capillaries were flushed with 0.2 M NaOH (20 min), water (10 min), and BGE (15 min) at 1 bar. Before each analysis, capillaries were flushed with 0.2 M NaOH (3 min), water

(1 min), MeOH (1 min), and BGE (5 min) at 1 bar. The injection, separation, and detection was at 50 mbar, +30 kV, and 210 nm, respectively.

### **3.3.3. Removal of $DS^-$**

The removal was performed using an Agilent CE (Waldbronn, Germany). Fused-silica capillary (Phoenix, AZ, USA) was 360  $\mu\text{m}$  o.d. and 200  $\mu\text{m}$  i.d.. The amount of micelles introduced electrokinetically into the capillary is proportional to the i.d. of the capillary. I.d.s larger than 200  $\mu\text{m}$  resulted in very high current and bubble formation inside the capillary. The length of the extraction capillary was 33 cm, which was the shortest possible length that could be accommodated by the capillary cartridge. New capillaries were flushed with 0.2 M NaOH (5 min), water (3 min), and ES (4 min) at 1 bar. Before each removal, capillaries were conditioned with 0.2 M NaOH (1 min), water (0.5 min), and ES (1 min) at 1 bar. The ES was 0.1M HCl in 40% ACN (apparent pH 2.5). The removal of  $DS^-$  was at a constant current of 175  $\mu\text{A}$  for 45 min. After each extraction at optimum conditions, negative pressure was applied (5 mbar, 1.2 s) to push back accumulated peptides at the tip of the capillary. At the end of each day, the electrodes were washed and stored in water.

The removal of  $DS^-$  with varying concentrations of SDS from 0.5 – 15 mM was studied. The removal time increased with increasing SDS concentration. In the removal of  $DS^-$  from samples after tryptic digestion, the final concentration of SDS was 3 mM, which required 45 min removal time and 40% ACN in the ES (optimum condition). 3mM SDS was also used in Sections 3.3 to 3.6. The % removal of  $DS^-$  was determined quantitatively by capillary zone electrophoresis (CZE) with indirect UV detection. The % removal was calculated as the CZE corrected peak area of  $DS^-$  after removal of  $DS^-$  divided by the corrected peak area before removal x 100%. The % removal of  $DS^-$  for 3mM SDS processed for 45 min was 99%.

### **3.3.4. Capillary zone electrophoresis with indirect UV detection of $DS^-$ .**

Fused-silica capillary was 33 cm in total and 24.5 cm to the detector. New capillary was flushed at 1 bar with 0.2 M NaOH (15 min), purified water (5 min), and separation solution (10 min). Capillaries before each run were flushed with 0.2 M NaOH (1 min), purified water (0.5 min), and separation solution (3 min). The sample was hydrodynamically introduced at 25 mbar for 10 s. Separation solution was 20 mM dihydroxybenzoic acid in 5% MeOH, adjusted to pH 9 with NaOH. Separation voltage and wavelength was -30 kV and 254 nm, respectively. The method was linear for SDS concentration range from 0.1 to 5.0 mM SDS in purified water with  $r^2 > 0.998$ . % RSD for 3 mM SDS (n=5) was 2.5 %.

### ***3.3.5. SDS denaturation and tryptic digestion.***

Protein sample (100 µg) was dissolved in 50 µL solution containing 100 mM tris-HCl, 10 mM DTT, and 1% by weight SDS. The solution was then incubated in a boiling water bath (5 min for low MW and 30 min for higher MW proteins). The solution was added with 10 µL of 100 mM IAA, and was stored in the dark for 1 hour. Finally, the mixture was diluted with 10 mM  $\text{NH}_4\text{HCO}_3$  to make 0.5 mg/mL or 0.1 mg/mL protein concentration. For the in-solution digestion, 10 µL of 0.5 mg/mL of trypsin prepared in 1 mM HCl was added and was incubated for 16 h at 37 °C. The digestion was quenched by addition of 10 µL of 25% acetic acid. The resulting concentration of SDS was 3 mM in both procedures.

### ***3.3.6. Direct infusion ESI-MS and data analysis.***

The ESI-MS of digested protein samples was performed on an Agilent 6320 Ion Trap system with an Agilent G1607A ESI interface. The sample was introduced using 75 µm i.d. capillary of 1 m total length at 1 bar. ESI voltage was +4 kV. Spray chamber parameters were as follows: nebulizer pressure 15.0 psi, dry gas flow-rate 5.0 L/min, drying gas temperature 325°C. Total ion scan was performed at  $m/z$  100 – 1200.

During the tryptic digestion, trypsin specifically cleaves at the carbonyl group followed either by arginine (Arg) or lysine (Lys) residue, except when Lys and Arg are N-linked to aspartic acid (Asp). In addition, hydrolysis will also not occur if proline is positioned on the carboxyl side of Lys and Arg <sup>16</sup>. Thus, the m/z of each theoretical peptide produced after tryptic digestion of a protein can be determined. An established algorithm was used to identify adduct ions formed after ESI-MS of digested protein samples <sup>17</sup>. The m/z of the theoretical peptide was entered in the algorithm and all possible adducts of the theoretical peptide is determined. MS signals of all adducts that belong to one molecular ion are added together, and are included in the calculation of % MS signal intensity (SI) restoration and % peptide recovery for protein digests.

### 3.4. Results and Discussion

#### 3.4.1. Removal of DS<sup>-</sup> through the electrophoretic separation of anionic DS<sup>-</sup> and cationic peptide

The removal of DS<sup>-</sup> in a digested protein sample with SDS can be achieved through the electrophoretic separation of negatively charged DS<sup>-</sup> and cationic peptides. The effective electrophoretic velocity of the peptides ( $v_{eff(peptide)}$ ) in the presence of SDS micelles is given by Eqn. (3.1) <sup>18</sup>.

$$v_{eff(peptide)} = \frac{1}{1+k} v_{ep(peptide)} + \frac{k}{k+1} v_{ep(micelle)} \quad (3.1)$$

$v_{ep(peptide)}$  and  $v_{ep(micelle)}$  are the electrophoretic velocities of the peptide and SDS micelle, respectively, while  $k$  is the retention factor.  $v_{ep(peptide)}$  or  $v_{ep(micelle)}$  is the product of the electrophoretic mobility ( $\mu$ ) of peptide or micelle directed towards the sample vial or the capillary, respectively, and electric field strength.

The  $v_{eff(peptide)}$  is negative or anodic in direction in the presence of SDS micelles. The peptides are solubilized into the negatively charged micelles, thus the  $k$  will be very high. The  $v_{eff(peptide)}$  is dictated by  $v_{ep(micelle)}$ . On the other hand, the  $v_{eff(peptide)}$  is positive in the absence of



SDS micelles, since the  $k$  will be zero. Micelles are formed at concentrations above the critical micelle concentration (cmc), the cmc of SDS is ~8 mM in water and lower (~2 mM) in the presence of salt <sup>19</sup>. In practice, removal of the surfactant is performed in the presence of salts, and thus SDS concentrations lower than 8 mM are accompanied by the presence of micelles in the sample. The use of organic solvents has been shown to reverse the  $v_{eff}$  of an analyte in the stacking technique of micelle to solvent stacking (MSS) in CE, causing the separation of cationic analytes and surfactants in a sample solution with SDS micelles <sup>20</sup>. The organic solvent collapses the micelles by increasing the cmc. To show the electrophoretic separation of  $DS^-$  and peptide, the CE analysis of bradykinin (model peptide) in the presence or absence of SDS micelles in the sample was therefore undertaken.

Figure 3.4.1.1 shows electropherograms of 20  $\mu$ M peptide in sample matrices with and without salt and SDS concentrations below and above the cmc. In Figure 3.4.1.1A and Figure 3.4.1.1B (without salt in sample), the BGE was 1 mM HAc in 25% MeOH. In Figure 3.4.1.1C and Figure 3.4.1.1D (with salt in sample), the BGE was 100 mM HAc. In Figure 3.4.1.1A and Figure 3.4.1.1C, the sample matrices were devoid of micelles. In Figure 3.4.1.1B and Figure 3.4.1.1D, the matrices had SDS micelles. The SDS concentration in Figure 3.4.1.1A and Figure 3.4.1.1B was 3.0 (<cmc) and 8.3 (>cmc) mM, respectively, in 1 mM HAc and 25% MeOH. The SDS in Figure 3.4.1.1C and Figure 3.4.1.1D was 0.5 (<cmc) and 3.0 (>cmc) mM, respectively, in 10 mM  $NH_4HCO_3$ . Sample injection was 60 s.

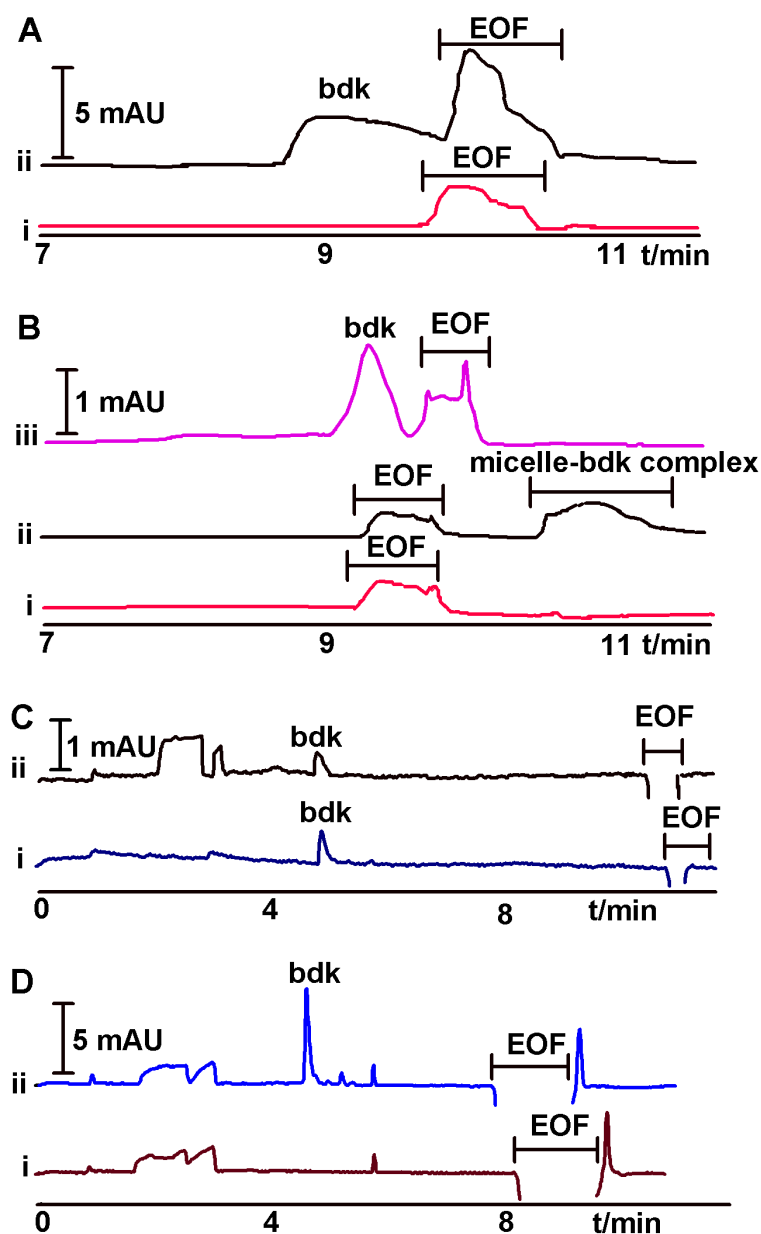


Figure 3.4.1.1. CE of bradykinin (bdk) as cationic species in sample matrices without (A and C) and with (B and D) SDS micelles. BGE: 1 mM HAc in 25% MeOH (A and B) and 100 mM HAc (C and D). Fused-silica capillary with 360  $\mu\text{m}$  o.d. and 50  $\mu\text{m}$  i.d. was used. The injection, separation, and detection was at 50 mbar, +30 kV, and 210 nm, respectively.

Figure 3.4.1.1Aii shows the detection of bradykinin before the electroosmotic flow (EOF), indicating a cathodic  $v_{\text{eff(peptide)}}$  for bradykinin. The  $\text{DS}^-$  migrated towards the anode. Figure 3.4.1.1Ai was an injection of sample matrix or blank, where the signal from the sample matrix was also the EOF marker. The EOF was verified by the injection of neutral acetone.

Figure 3.4.1.1Bii shows the detection of bradykinin (as the micelle-bradykinin complex) after the EOF, suggesting an anodic  $v_{eff(peptide)}$ . Figure 3.4.1.1Bi was a blank injection.  $DS^-$  removal is therefore difficult at concentrations above the cmc<sup>12</sup>. However, the separation of peptide and  $DS^-$  can be achieved by MSS, as shown in Figure 3.4.1.1Biii. MSS was by injection of 40% ACN in 1 mM HAc (60 s) after the sample. In the presence of an electric field, the micelle–bradykinin complex migrated to the anode, and into the ACN zone. The micelles collapsed at the boundary between the micelles and ACN-rich zones, releasing the peptide. The  $v_{eff(peptide)}$  of bradykinin reversed from anodic to cathodic in direction. Bradykinin was detected before the EOF. MSS could therefore be implemented for electrokinetic  $DS^-$  removal at concentrations above the cmc.

Figure 3.4.1.1Cii was an injection of bradykinin in SDS below the cmc and in the presence of salt. bradykinin was detected before the EOF, suggesting the separation of bradykinin from  $DS^-$ . This is a result similar to Figure 3.4.1.1Aii. bradykinin was verified by injection of standard sample without SDS (see Figure 3.4.1.1Ci). The peaks before 4 min in Figure 3.4.1.1Cii were system peaks generated from the sample matrix, which was verified by blank injection. The result in Figure 3.4.1.1Cii suggests that electrophoretic removal of  $DS^-$  can be easily achieved at concentrations below cmc, even in the presence of salt.

In Figure 3.4.1.1Di, bradykinin was not detected before the EOF because of the presence of micelles at low SDS concentration in the matrix with salt. It is noted that bradykinin was detected at negative polarity separation (-30 kV), suggesting the anodic apparent migration of micelle-bradykinin complex. However, by MSS (60 s injection of 40% ACN in 100 mM HAc), bradykinin was detected before the EOF and separated from  $DS^-$  as shown in Figure 3.4.1.1Dii. This is the fundamental basis of this work, which is to demonstrate the electrokinetic removal of  $DS^-$  in samples that contain SDS above the cmc and

in the presence of salt. The removal of  $DS^-$  from micellar sample solutions and in the presence of salts is more challenging and relevant to real-world samples.

### **3.4.2. Selective removal of $DS^-$ and recovery of peptide**

The general scheme for electrokinetic  $DS^-$  removal in the presence of SDS micelles in the sample solution is as follows. An acidic ES in 40% ACN was housed inside a 200  $\mu\text{m}$  i.d. capillary. Higher %s of ACN caused bubble formation inside the capillary. A vial containing the sample solution (50  $\mu\text{L}$ ) was placed at the inlet end of the capillary, and a vial containing ES was placed at the outlet end (Figure 3.4.2.1A). The removal of  $DS^-$  was at a constant current of 175  $\mu\text{A}$  for 45 min. Higher currents caused bubbling in the sample due to excessive heat generation. A constant current also provided better reproducibility than constant voltage mode. Decreasing the current to accommodate higher concentration of ACN, e.g. 50%, increased the removal time, which was not practical. The  $DS^-$  monomers and micelles migrated into the capillary under the applied electric field with the cathode being placed at the sample vial (Figure 3.4.2.1B). The electrophoretic velocities of the micelles and the monomers,  $v_{DS^- (\text{micelles})}$  and  $v_{DS^- (\text{monomers})}$ , respectively, were greater than the velocity of the EOF,  $v_{eof}$ , owing to the acidic conditions in the capillary.  $v_{eof}$  was directed towards the sample vial.

At the MSS boundary (MSSB) located at the tip of the capillary in contact with the sample solution, the presence of the organic solvent collapsed the micelles<sup>21</sup>; releasing the peptides bound to these micelles through MSS mechanism<sup>20</sup>. The high ACN content in the ES also prevented possible electrostatic interactions between peptides and  $DS^-$  monomers. It is well documented that there is also an electric field in the sample vial that allows electrokinetic injection of charged species<sup>22</sup>. The removal of  $DS^-$  was accompanied by the generation of hydroxide ions from the electrolysis of water at the sample vial, which maintains the electroneutrality. The peptides were accumulated at the tip of the capillary, while the  $DS^-$  monomers travelled towards the positive electrode. The positively charged

peptide molecules were therefore retained at the tip of the capillary due to the continued flux of  $\text{DS}^-$  from the sample vial. The voltage was stopped and applied pressure was then necessary to push the stacked peptides back to the sample vial for ESI-MS analysis (Figure 3.4.2.1C).

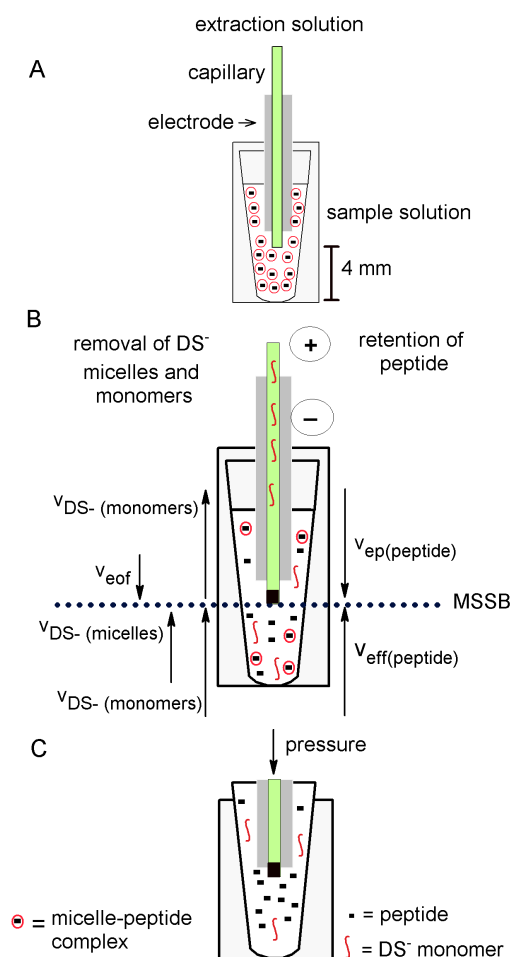


Figure 3.4.2.1. Mechanism of the selective removal of  $\text{DS}^-$ . (A) was the initial condition. The sample solution contained peptides (black dots) and  $\text{DS}^-$  micelles (red circles). The capillary was filled with acidic ES with ACN. The sample and the ES vials were placed at the cathodic and anodic ends of the capillary, respectively. (B) Voltage was applied with the cathode at the sample vial. The  $\text{DS}^-$  monomers and micelles were introduced into the capillary. (C) Voltage was stopped and the stacked peptides were pushed back to the sample vial by pressure.

### 3.4.3. Proof of concept – selective removal of $\text{DS}^-$ and recovery of peptide using ACN in the ES

Figure 3.4.3.1 shows the % MS SI restoration of ZGGV and the % MS SI reduction of  $\text{DS}^-$  with 0–40% ACN and 0.1 M HCl in the ES. The sample contained 3 mM SDS ( $>\text{cmc}$ ), 100  $\mu\text{M}$  ZGGV, and 10 mM  $\text{NH}_4\text{HCO}_3$ . % MS SI restoration of the analyte ( $n=5$ , %RSD =3.4) was equal to MS SI of peptide after  $\text{DS}^-$  removal / MS SI from standard without SDS  $\times 100\%$ . % MS SI reduction of  $\text{DS}^-$  was obtained by dividing the difference of the  $\text{DS}^-$  ( $m/z$  311 or 599) signals before and after treatment by the SI before treatment, multiplied by 100%. We note that the calculated % MS SI reduction using  $m/z$  311 and  $m/z$  599, which is another signal for  $\text{DS}^-$ , were comparable. Here, % MS SI reduction of  $\text{DS}^-$  was based primarily using  $m/z$  311.

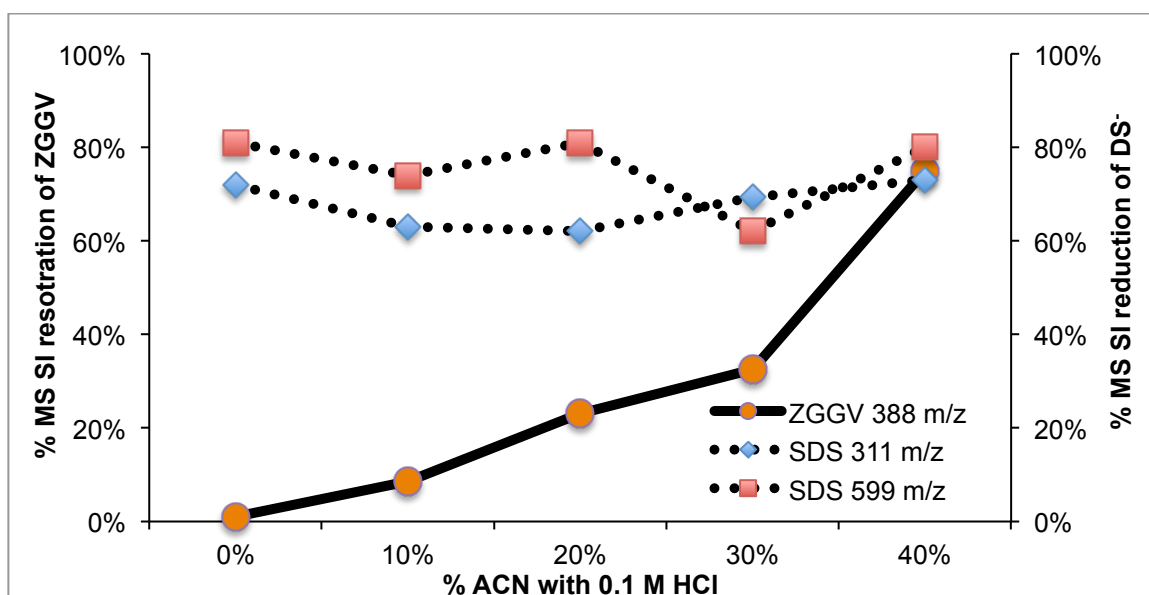


Figure 3.4.3.1. % MS SI restoration of ZGGV and % MS SI reduction of  $\text{DS}^-$ . The removal of  $\text{DS}^-$  was at a constant current of 175  $\mu\text{A}$  for 45 min, with no back-pressure step. The removal was performed using an Agilent CE and the fused-silica capillary was 200  $\mu\text{m}$  i.d. and 33 cm total length. The ES was 0.1M HCl in 40% ACN (apparent pH 2.5).

Increasing % MS SI restoration of the peptide was observed as the concentration of ACN was increased. At 0% ACN, 1% MS SI restoration of ZGGV resulted and this increased to 9%, 23%, and 33% when 10, 20, and 30% ACN was in the ES, respectively. The peptide was bound to the micelles at these %ACN, leading to the removal of the peptides from the sample vial into the capillary as micelle-peptide complexes. At 40% ACN, however, a

significant increase of the % MS SI restoration of ZGGV (75%) was observed. At this %ACN, the micelles collapsed at the MSSB, thereby releasing the peptides at the capillary tip. On the other hand, the % MS SI reduction of DS<sup>-</sup> was 62 – 81% for m/z 599 and 62 – 73% for m/z 311, and therefore, DS<sup>-</sup> removal was unaffected by the %ACN in the ES. All DS<sup>-</sup> species in the sample entered the capillary and travelled towards the anode, and this migration direction was unaffected by ACN. Figure 3.4.3.2 shows the representative ESI-MS spectra for the removal of DS<sup>-</sup> and recovery of ZGGV.

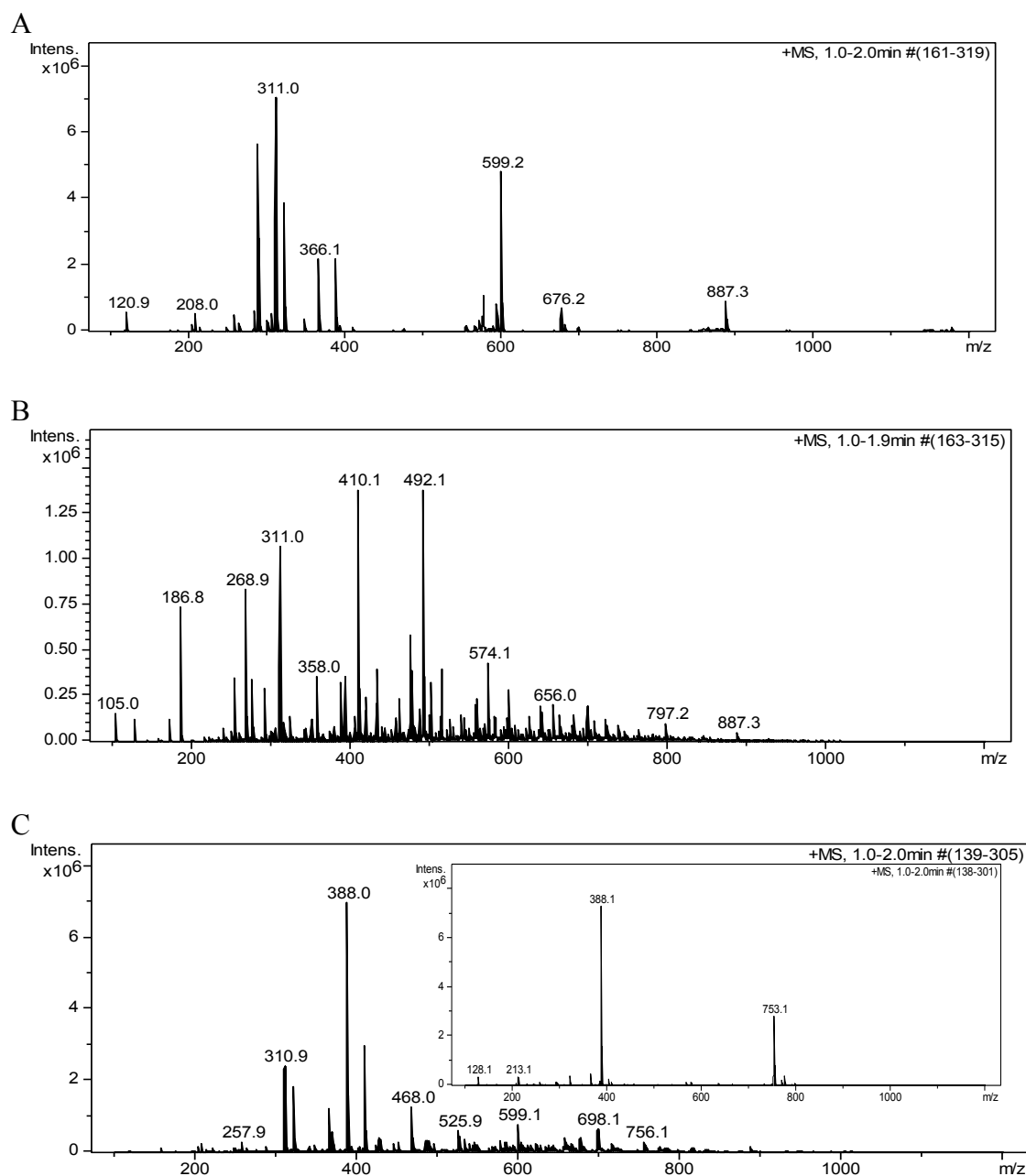


Figure 3.4.3.2. ESI-MS spectra of (A) before DS<sup>-</sup> treatment, and (B) and (C) after treatment of 100  $\mu$ M ZGGV, 3 mM SDS, 10 mM NH<sub>4</sub>HCO<sub>3</sub> with an inset spectra of 100  $\mu$ M ZGGV standard. Extraction solution used in (B) was 0.1 M HCl, while (C) was 0.1 M HCl in 40% ACN. The removal was performed using an Agilent CE, the fused-silica capillary was 200  $\mu$ m i.d. and 33 cm total length, and at constant current of 175  $\mu$ A.

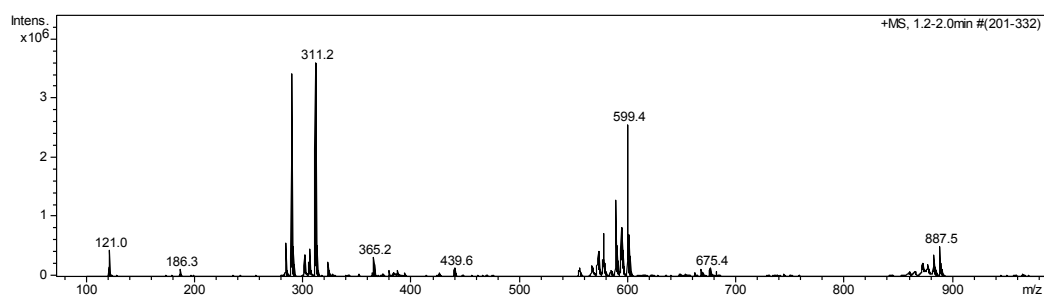


#### ***3.4.4. Optimization of removal time and back-pressure***

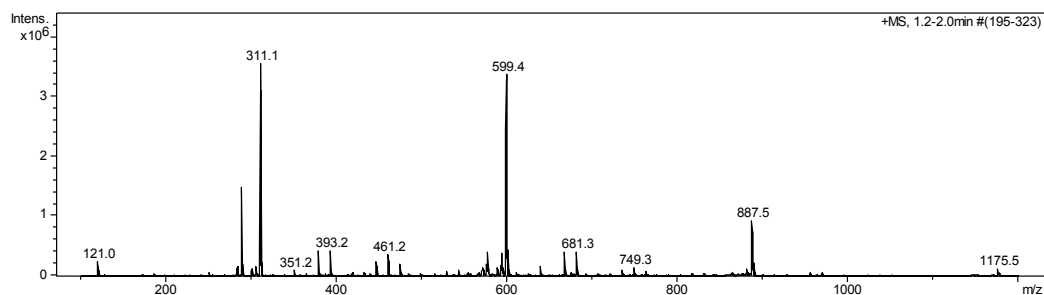
Increasing DS<sup>-</sup> removal time (15, 30, 45 min) from a solution containing 3 mM SDS and 10 mM NH<sub>4</sub>HCO<sub>3</sub> was conducted. Figure 3.4.4.1 depicts the MS SI reduction of DS<sup>-</sup> when the removal time was increased. The % MS SI reduction of DS<sup>-</sup> (m/z 311) was 88% after 45 min removal time.

We have recently visualized the stacking by MSS of a cationic fluorescent rhodamine B using a single straight channel microchip CE device <sup>23</sup>. Rhodamine B in an acidic micellar SDS solution was placed in the sample reservoir, while the channel and outlet reservoir were filled with organic solvent-rich low pH solution. With a negative voltage applied at the sample reservoir, the probe stacked at the tip of the channel adjacent to the sample. This configuration was similar with our DS<sup>-</sup> removal system, and the results further justify the need to study the back-pressure after electrokinetic removal. After optimization, the highest % MS SI restoration of the peptide was obtained when 5 mbar was applied for 1.2 s. After 45 min removal time, SDS micelles were depleted from the sample; however, there is the possibility that some DS<sup>-</sup> monomers may be left in the sample vial. These monomers can enter the capillary and migrate towards the anode, but this process will not mobilise the peptide accumulated at the capillary tip.

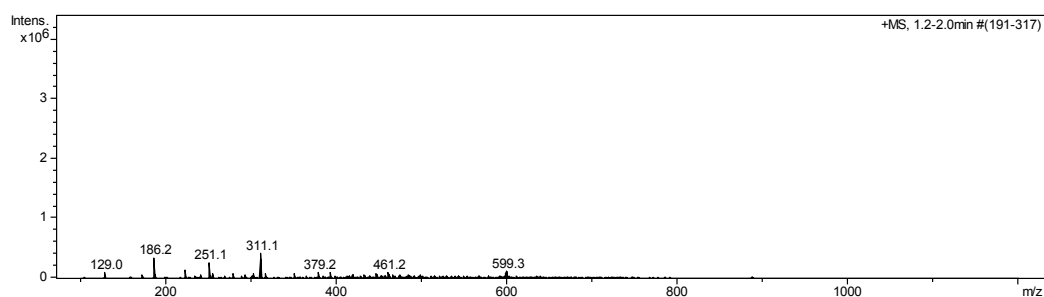
A.



B.



C.



D.

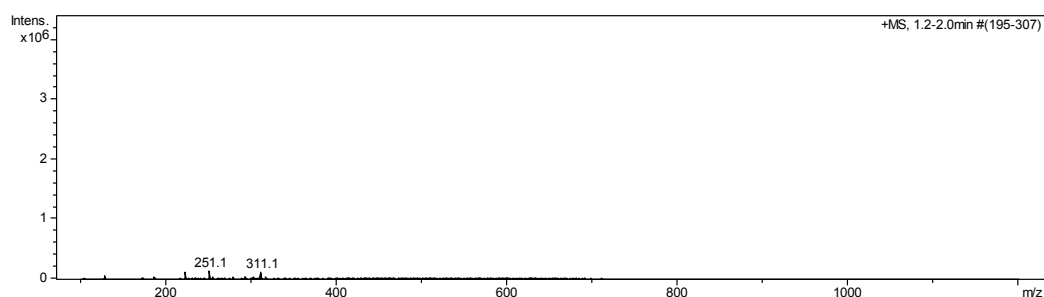


Figure 3.4.4.1. DS<sup>-</sup> removal at 175 uA for 0 (A), 15 (B), 30 (C), and 45 (D) min from solution containing 3 mM SDS and 10 mM NH<sub>4</sub>HCO<sub>3</sub>. Extraction solution, 0.1 M HCl in 40% ACN. The removal was performed using an Agilent CE and the fused-silica capillary was 200  $\mu$ m i.d. and 33 cm total length.

### 3.4.5. Application to larger peptides

The selective removal of  $\text{DS}^-$  and MS SI restoration of peptide was further assessed using bradykinin (molar mass =1062 g/mol). Fig. 4 shows the ESI-MS spectra before (A) and after (B) removal of  $\text{DS}^-$  from a sample containing 20  $\mu\text{M}$  bradykinin in 3 mM SDS and 10 mM  $\text{NH}_4\text{HCO}_3$ . Without removal, high SI of  $\text{DS}^-$  dominated the spectrum, suppressing the bradykinin signals (e.g., at  $m/z$  530.7). 99% MS SI reduction of  $\text{DS}^-$  ( $m/z$  311) was achieved. This value was similar with the % removal of  $\text{DS}^-$  = 99% (by CZE with indirect UV). The bradykinin signals were visible with 83% MS SI restoration ( $n=5$ , %RSD =2.7) as shown in Fig. 4B. The slightly lower value for % MS SI restoration compared to the % recovery (by MEKC-UV) was due to the effect of SDS in ESI-MS. Signals marked with asterisks are bradykinin signal adducts with  $m/z$  362 = $[\text{M}+2\text{H}+\text{Na}]^{3+}$ , 542 = $[\text{M}+\text{H}+\text{Na}]^{2+}$ , 552 = $[\text{M}+\text{ACN}+2\text{H}]^{2+}$ , 554 = $[\text{M}+2\text{Na}]^{2+}$  which were included in the calculation of % MS SI restoration. The summation of the SIs of adducts from the same molecular ion has been used for quantitative analysis of the molecular ion in ESI-MS<sup>24,25</sup>. The detection limit using the approach would be very close to that from direct infusion ESI-MS.

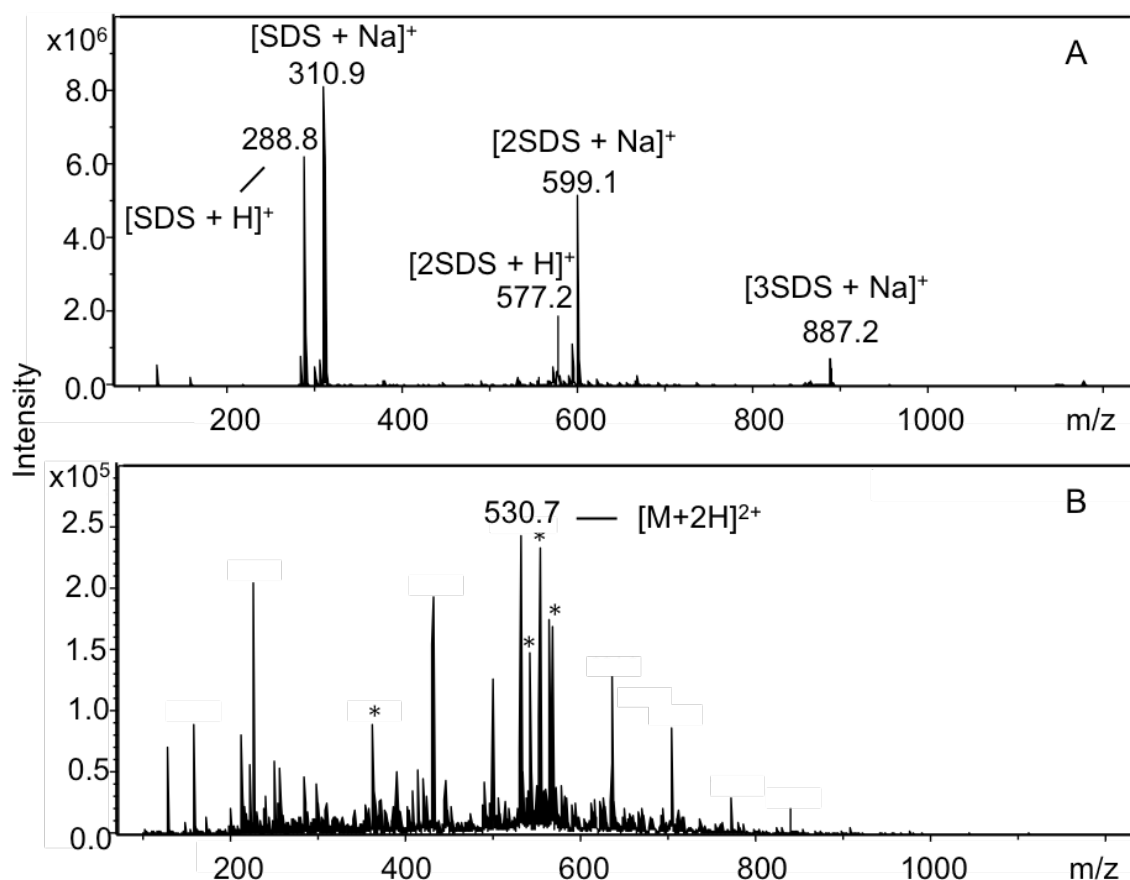


Figure 3.4.5.1. Representative ESI-MS spectra before (A) and after (B) electrokinetic removal of 20  $\mu$ M bradykinin, 3 mM SDS, 10 mM  $\text{NH}_4\text{HCO}_3$  sample. Signals with asterisks (\*) are also for bradykinin. Removal conditions: the same as Figure 3.4.3.2.

### 3.5. Conclusion

Selective removal of  $\text{DS}^-$  from micellar sample solutions that contain peptides or digested proteins is proposed. The  $\text{DS}^-$  removal was driven by the electric field and the peptide recovery was facilitated by MSS. The amount of organic solvent (i.e. 40% ACN) in the ES is important for the recovery, but not for  $\text{DS}^-$  removal. The leakage of ES into the sample vial (10% increase in sample volume) explains the %recoveries obtained that were not >90%. The method could process 1 sample in 48 min including conditioning steps using a single capillary CE device. This translates to 30 samples a day, since the CE instrument could be programmed

to run in sequence. The approach is simple and minimized the use of organic solvent compared to commonly used techniques for SDS removal in protein and peptide samples.

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## Chapter 4

### **Electrokinetic device integrated to electrospray-ionization mass spectrometry for the simultaneous removal of sodium dodecyl sulfate and enrichment of peptides**

#### **4.1. Chapter Summary**

The removal of sodium dodecyl sulfate (SDS) in SDS-assisted proteomics with electrospray ionization mass spectrometric (ESI-MS) analysis is an essential step in the analysis. Off-line state-of-the-art sample preparation strategies can allow 100% removal of DS<sup>-</sup> and up to 100% peptide recoveries. These strategies, however, are typically laborious and require long analysis times and a complex experimental set-up. Here, we developed a simple electrokinetic on-line integrated SDS removal – ESI-MS device that was able to improve ESI-MS signals of bradykinin and peptides from tryptic digested bovine serum albumin (BSA) in samples that contain SDS micelles. The significant peptide signal improvements were contributed by the removal of DS<sup>-</sup> and enrichment of the peptides in the presence of an electric field. Enrichment was via micelle-to-solvent stacking, initially developed in capillary electrophoresis. The percent MS signal intensity (SI) restoration of bradykinin and BSA peptides were 800% and 87%, respectively. Improvement factors in ESI-MS signals (after and before removal) for selected  $m/z$  values of BSA peptides after digestion were 535 – 693.



## 4.2. Introduction

Efficient sample preparation is a vital step of global proteomics, especially when the analysis involves the ultimate elucidation of peptides and proteins by electrospray ionisation-mass spectrometry (ESI-MS). In the tryptic digestion process in bottom-up proteomics, solubilization and digestion of membrane proteins requires additives, such as sodium dodecyl sulfate (SDS). The strong binding of SDS with proteins is due to the highly hydrophobic nature of membrane proteins and SDS, resulting in the formation of SDS-protein complexes<sup>1</sup>. Solubilization results in disaggregation and unfolding of proteins and thus allows access of protease to cleavage sites during tryptic digestion. Unfortunately, the presence of SDS in the prepared sample solution is detrimental to MS analysis due to the production of  $DS^-$  signals that dominate the spectra<sup>2,3</sup> and also cause signal reduction of peptides. Removal of SDS is therefore an essential prerequisite prior to ESI-MS analysis in order to obtain high coverage of peptides that ensures high confidence protein identification.

Removal of SDS prior to ESI-MS has been accomplished by a variety of off-line techniques such as strong cation-exchange<sup>4</sup>, membrane-based methods (e.g. filter-aided sample preparation<sup>5</sup>), and peptide or surfactant precipitation<sup>6,7</sup>. In order to mitigate the issue of membrane-fouling in membrane-based methods, Kachuk and co-workers<sup>8</sup> used an electric-field in conjunction with membranes. They easily removed SDS after one hour from 400  $\mu$ L protein standards and membrane fractions from *E. coli* samples. Very high removal (99.9%) of surfactant and recovery of analytes were attained.

Direct infusion ESI-MS is a simple and rapid approach of peptide analysis for protein identification<sup>9,10</sup>. Tedious salt and SDS removal is however required prior to direct infusion. SDS removal systems integrated with ESI-MS (integrated SDS removal – ESI-MS) should increase sample throughput and minimize sample loss, which are desired in proteomic workflows. Despite these advantages, integrated systems for SDS removal are scarce with

only one paper reported <sup>11</sup>. Efficient removal of SDS is the basic requirement in integrated SDS removal – ESI-MS, since even small concentrations of SDS (i.e. >0.3 mM) are detrimental to ESI-MS <sup>12</sup>. Kinde, et al. developed a microfluidic device integrated in-line to ESI –MS and the system was utilized for the efficient removal of up to 10 mM SDS from a bradykinin standard solution prepared in 25% methanol, but not for protein digested samples <sup>11</sup>. Micelle-to-solvent stacking (MSS) is a sample enrichment technique in capillary electrophoresis (CE) that releases and concentrates micelle-bound analytes at a boundary inside the capillary between a micellar solution and an organic solvent-rich zone <sup>13</sup>. SDS removal with the aid of MSS was previously demonstrated using a commercial CE instrument as an off-line sample preparation approach. Percent MS signal intensity (SI) restoration of the peptide were 74-83% and percent MS SI reduction of DS<sup>-</sup> with 89-95% were achieved, and higher peptide %recoveries for digested proteins compared to SDS removal by cold acetone precipitation were obtained. The method could process 1 sample in 48 min, excluding the time required for the ESI-MS analysis itself.

Here, we report an electrokinetic on-line integrated SDS removal – ESI-MS device, which is capable of significantly improving peptide signals and potentially, also sample throughput. The overall scheme of the on-line integrated SDS removal – ESI-MS is presented in Figure 4.4.1.1. The simple device is composed of a pressure source, voltage source, and multi-capillary assembly. The multi-capillary assembly is made up of fused-silica capillaries of different dimensions (see Figure 4.4.1.1A, sample, extraction, extraction solution (ES), and MS capillaries) connected using leak-free interconnect tees. A small volume of ~3.5  $\mu$ L micellar SDS sample solution was loaded into the sample capillary using the pressure source. An electric field and acetonitrile (ACN)-rich ES in the extraction capillary were used to enrich the peptides, and also separate the SDS from the enriched peptides. The MSS-enriched peptides were directly infused by pressure to the ESI-MS through the MS capillary. The

performance of the multi-capillary assembly was primarily dependent on the dimensions of the extraction capillary. Thus, the extraction capillary was optimized by varying the length and internal diameter (i.d.). The  $\text{DS}^-$  removal was optimized by varying the voltage, voltage application time, and simultaneous voltage and pressure application time. These optimization experiments were conducted using bradykinin as model peptide in 3 mM SDS and 10 mM  $\text{NH}_4\text{HCO}_3$  (above the cmc of SDS<sup>14</sup>). After optimization, the device was applied to the analysis of tryptic digested bovine serum albumin (BSA).

### **4.3. Materials and Methods**

#### **4.3.1. Reagents and Stock Solutions**

Sodium hydroxide (NaOH, >95%), acetic acid (HAc), hydrochloric acid (HCl), acetonitrile (ACN, HPLC grade), methanol (MeOH), sodium dodecyl sulfate (SDS), dihydroxybenzoic acid (DHBA), Tris-HCl, 1,4-dithiothreitol, iodoacetamide (IAA), ammonium bicarbonate ( $\text{NH}_4\text{HCO}_3$ ), trypsin from bovine pancreas were obtained from Sigma-Aldrich (St. Louis, MA) or Fluka (Buchs, Switzerland). Bradykinin and bovine serum albumin BSA (69 kDA) were purchased from Sigma-Aldrich. Purified water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). Stock solution of the peptide was 200  $\mu\text{M}$  in purified water. ES was 5 M HAc in 40% ACN. The mobilization solution was 50 mM  $\text{NH}_4\text{HCO}_3$ .

Different percentages of ACN (0 – 60) in ES were investigated. Substantial recoveries of peptide and removal of  $\text{DS}^-$  were obtained when  $\geq 40\%$  ACN was used, consistent with the previous study<sup>14</sup>.

#### **4.3.2. SDS denaturation and tryptic digestion.**

Protein sample (100 µg) was dissolved in 50 µL solution containing 100 mM tris-HCl, 10 mM DTT, and 1% by weight SDS. The solution was then incubated in a boiling water bath (5 min). The solution was added with 10 µL of 100 mM IAA, and was stored in the dark for 1 hour. Finally, the mixture was diluted with 10 mM  $\text{NH}_4\text{HCO}_3$  to make 0.5 mg/mL or 0.1 mg/mL protein concentration. For the in-solution digestion, 10 µL of 0.5 mg/mL of trypsin prepared in 1 mM HCl was added and was incubated for 16 h at 37 °C. The digestion was quenched by addition of 10 µL of 25% acetic acid. The resulting concentration of SDS was 3 mM in both procedures.

#### **4.3.3. Electrokinetic on-line integrated SDS removal – ESI-MS device design.**

Figure 4.3.3.1 shows the photo of the multi-capillary assembly with the sample capillary connected to the pressure source. The pressure source was from PrinCE model 250 (Emmen, Netherlands) and the voltage source was from Matsusada model AU-30P1-L (Shiga, Japan). Polyimide-coated fused-silica capillaries of different i.ds. and 360 µm o.d. were obtained from Polymicro (Arizona, USA). The multi-capillary assembly was composed of the following: (1) sample capillary (100 µm i.d, 40 cm total length), (2) MS capillary (100 µm i.d, 40 cm total length), (3) extraction capillary (200 µm i.d, 5 cm total length), (4) ES capillaries (25 µm i.d, 15 cm total length each). All capillaries were connected to each other using (5) interconnect tees (400 x 400 mm; Labsmith, USA) and were secured using one-piece PEEK fittings. 15 cm total length of the ES capillaries was chosen because this was the minimum length that could be used most conveniently in the set-up. 25 µm i.d. material was used for the ES capillaries in order to restrict the flow of the sample matrix towards the ES vials. 40 cm total lengths of the sample and MS capillaries were chosen because this was the minimum comfortable length from the pressure source and the MS instrument, respectively.

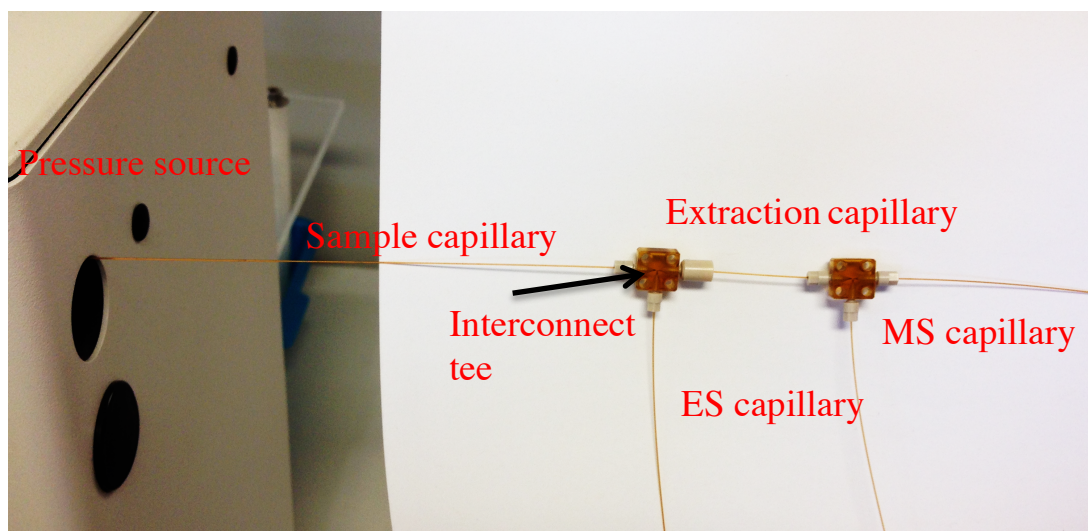


Figure 4.3.3.1. Photo of the multi-capillary assembly with the pressure source

The sample capillary was connected to the pressure source for sample loading, while the MS capillary was directly attached to the ESI. The two ES capillaries were dipped into the vials filled with ES (ES vials). Voltage was applied with the anode and cathode to the vial closer to the ESI-MS and vial closer to the sample capillary, respectively.

#### 4.3.4. *General procedure.*

The ESI-MS was put on standby. New capillaries used in the multi-capillary assembly were flushed with 0.2 M NaOH (20 min) and water (5 min) using a handheld syringe pump. In between runs, the multi-capillary assembly was flushed with 0.2 M NaOH (6 min) and water (6 min) at 50 mbar from the sample capillary. 5.5 min was required to fill the sample, extraction, and MS capillaries during continuous pressure application. The multi-capillary assembly was filled with the ES, which was introduced from the ES capillary near the ESI-MS using a handheld syringe pump. Care was taken to ensure that there were no air bubbles in the assembly.

The sample capillary was loaded with the sample solution (50 mbar for 2 min, ~3.5  $\mu$ L). The sample vial was then replaced with the mobilization solution and pressure was applied (50 mbar for 2 min). During this step, voltage (10 kV) was applied at the ES vials.

The dissipation of the electric field towards the MS capillary was avoided by putting the ESI-MS on standby, which also resulted in repeatable  $\text{DS}^-$  removal (%RSD <10%,  $n = 10$ ). A fraction of the sample solution flowed towards the ES vial near the sample capillary, where the EOF was also directed. The other fraction remained in the sample capillary. The pressure was stopped, leaving the voltage on for 10 min, which resulted in high  $\text{DS}^-$  removal from the sample fraction remaining in the sample capillary. The voltage was then turned off, the MS instrument was turned on, and pressure (50 mbar) was applied to directly infuse the enriched peptides into the ESI-MS. The entire optimized clean-up process was 12 min in total, excluding flushing.

#### **4.3.5. ESI-MS and data analysis.**

The ESI-MS was performed on an Agilent 6320 Ion Trap system with an Agilent G1607A ESI interface. ESI voltage was +4 kV. Spray chamber parameters were as follows: nebulizer pressure 15.0 psi, dry gas flow-rate 5.0 L/min, drying gas temperature 325°C. Total ion count (TIC) scan was at  $m/z$  100 – 1500. Identification of peptides and adducts was performed as described previously [14].

### **4.4. Results and Discussion**

#### **4.4.1. Mechanism of $\text{DS}^-$ removal and peptide enrichment**

Figure 4.4.1.1B depicts the mechanism for the simultaneous  $\text{DS}^-$  removal and peptide enrichment. An MSS boundary (MSSB) was formed between the sample solution and the ES, within the interconnect tee after sample injection. The  $\text{DS}^-$  removal and peptide enrichment occurred during the simultaneous application of pressure and voltage (Figure 4.4.1.1Bi), and voltage only (Figure 4.4.1.1Bii). In the presence of an electric field, the SDS micelles and SDS-bound peptides were electrokinetically injected into the interconnect tee from the sample capillary. The electrophoretic velocity of micelles,  $v_{ep(\text{micelles})}$ , and the effective electrophoretic velocity of peptides,  $v_{eff(\text{peptides})}$ , were towards the anode or extraction

capillary. At the MSSB, the micelles collapsed, releasing the peptides. The velocity of the released peptides,  $v_{ep(peptides)}$ , was opposite the  $v_{eff(peptides)}$ . The peptides were re-captured by the electrokinetically injected micelles. The collapse and the recapture (effective electrophoretic mobility reversal in MSS) caused the enrichment of the peptides until all the micelles were exhausted<sup>13</sup>. The velocity of the  $DS^-$  monomers,  $v_{DS^-(monomers)}$  was to the anode, causing the removal of the  $DS^-$  from the sample. Figure 4.4.1.1.C shows the application of pressure until the enriched peptides without  $DS^-$  were detected by the ESI-MS.

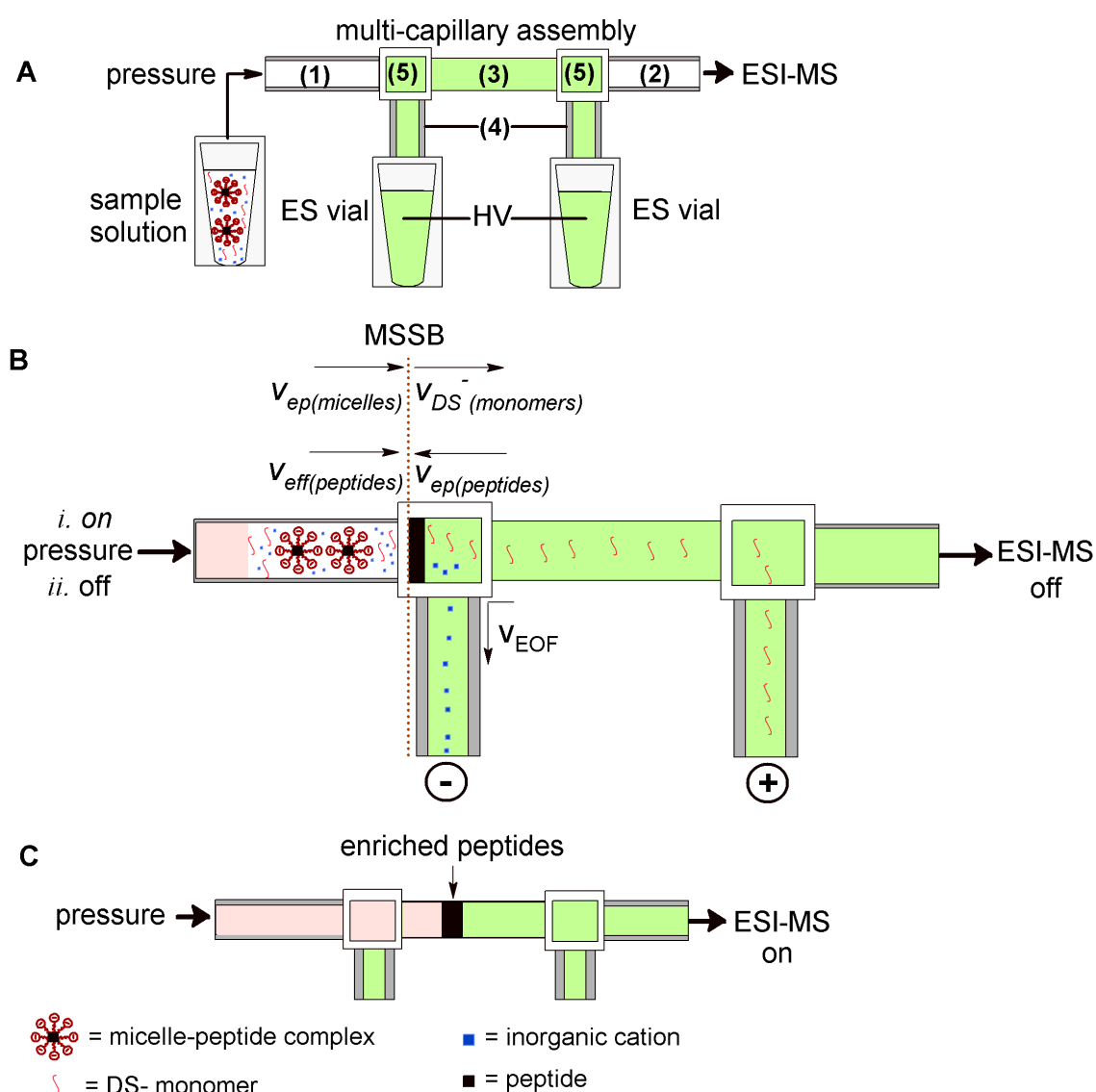


Figure 4.4.1.1. Overall scheme of  $DS^-$  removal and peptide enrichment. (1) sample capillary, (2) MS capillary, (3) extraction capillary, (4) ES capillaries, (5) interconnect tees.

#### 4.4.2. Proof of concept

Figure 4.4.2.1A shows an ESI-MS spectrum of the sample (bradykinin in 3 mM SDS with 10 mM  $\text{NH}_4\text{HCO}_3$ ) without electrokinetic removal of  $\text{DS}^-$ . Consistently high signals for SDS and adducts ( $m/z$  311.1, 599.4, 887.5), which dominated the MS spectrum, were observed from 1.5 – 5.5 min (see Figure 4.4.2.1A1). Figure 4.4.2.1D shows the electrokinetic on-line integrated SDS removal – ESI-MS spectrum of the sample. The enriched bradykinin was detected by the MS after 5.5 min. A lag from 1 – 2 min was observed for the detection of the enriched bradykinin. This was attributed to the presence of air bubbles generated during electrokinetic removal, which reduced the liquid flow during direct infusion into the ESI-MS. SDS signals were absent from the ESI-MS spectra, and only a prominent signal at  $m/z$  =531 for bradykinin was observed (see Figure 4.4.2.1D1), which indicated the complete removal of  $\text{DS}^-$ .

The effect of voltage application time from 0 to 12 min for electrokinetic removal (including the 2 min voltage time from the simultaneous pressure and voltage application) was investigated to monitor the removal of  $\text{DS}^-$  and the enrichment of peptide. The results for 4, 9, and 12 min are shown in Figure 4.4.2.1B, C, and D, respectively, where D was also discussed above. With the time of 9 min (C), the SDS zone was shorter compared to the 4 min (B). This was because more  $\text{DS}^-$  monomers were removed from the extraction capillary and into the ES vial at the anode. In B, the extraction capillary was filled by the  $\text{DS}^-$  monomers. Enriched peptides (\*) were observed, but were not free of SDS. In C, there was a small zone of  $\text{DS}^-$  monomers adjacent to the enriched bradykinin. The enriched peptide zone was not free of SDS and caused some bradykinin ESI-MS signal suppression. In summary, this study of voltage application time provided insights on the removal of  $\text{DS}^-$  from the sample, and 12 min (D) was required for complete  $\text{DS}^-$  removal and peptide enrichment in our



present system. It is necessary to optimize the voltage application time to maximize the ESI-MS signal of the peptides.

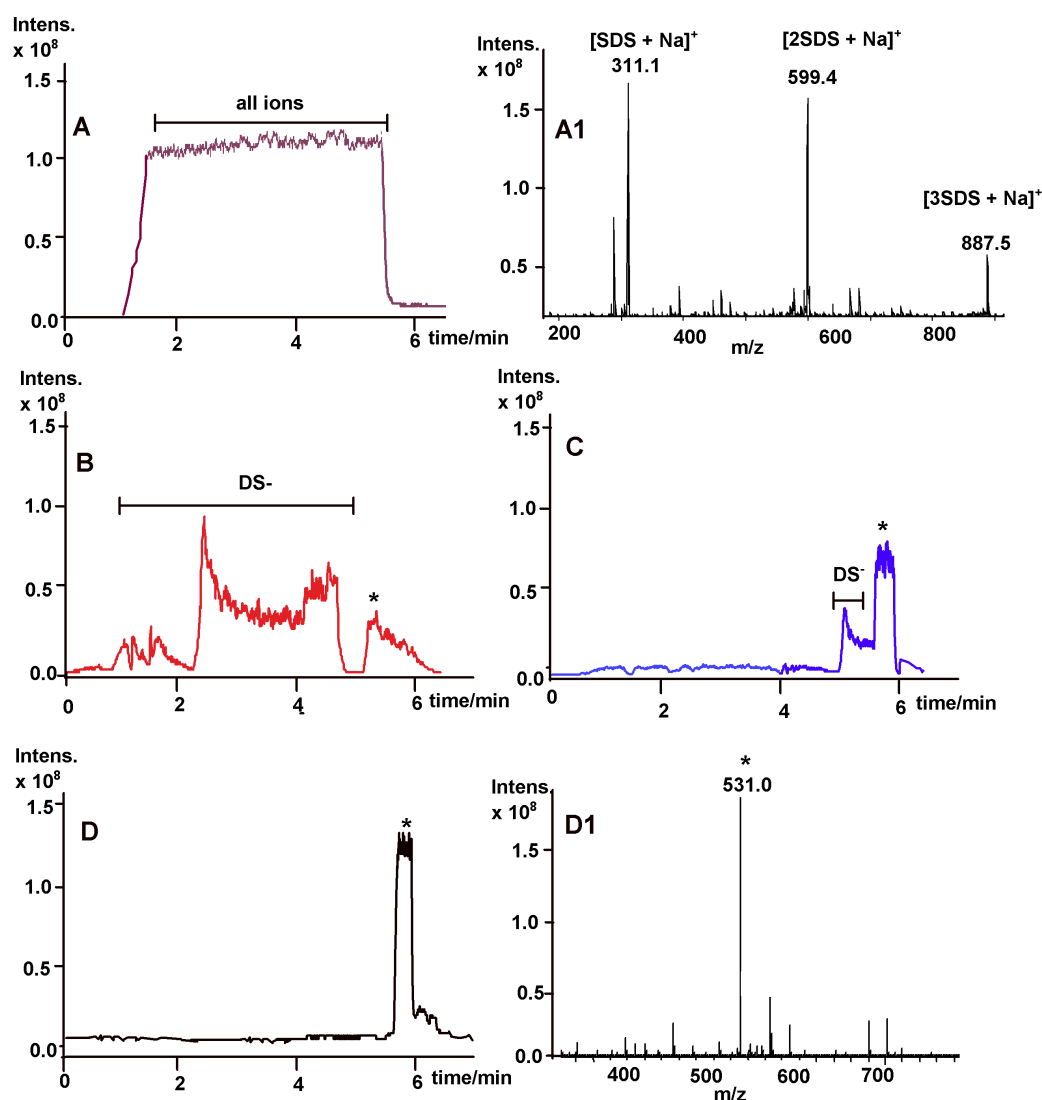


Figure 4.4.2.1. ESI-MS total ion count (TIC) trace obtained during direct infusion of 20  $\mu\text{M}$  bradykinin in 3 mM SDS and 10 mM  $\text{NH}_4\text{HCO}_3$  sample without (A), and with electrokinetic on-line integrated SDS removal – ESI –MS (B-D). Voltage application times were 0 (A), 4 (B), 9 (C), 12 (D) min.

Percent MS SI restoration was determined by dividing the ESI-MS signal of bradykinin after  $\text{DS}^-$  removal by the signal of bradykinin in a standard solution without  $\text{DS}^-$ , multiplied by 100%. The % MS SI restoration for bradykinin was 800%, or a signal enrichment factor of 8. In the literature, % MS SI restoration of peptides were typically up to 100% only<sup>12</sup>. The very

high recovery of our method was a result of the enrichment of the peptide during MSS and the removal of DS<sup>-</sup>.

#### **4.4.3. Optimization**

This section describes the optimization of the dimensions of the extraction capillary, applied voltage across the extraction and ES capillaries, and simultaneous pressure and voltage application time. For the extraction capillary, different i.ds. (50, 75, 100, 150, 200, 250  $\mu\text{m}$ ) and lengths (5, 10, 15, 20 cm) of the capillary were investigated. At a fixed length of 10 cm, the highest peptide recovery was obtained with 200  $\mu\text{m}$  i.d. capillary. This is because the total amount of ions that can be accommodated in a capillary during electrokinetic injection is directly proportional to the capillary i.d.<sup>15, 16</sup>. The 250  $\mu\text{m}$  i.d. capillary, however, was very brittle and broke easily during voltage application. Then, with a 200  $\mu\text{m}$  i.d. extraction capillary, the highest peptide recovery was obtained with 5 cm length. This was due to the higher electric field strength ( $E = \text{applied voltage}/\text{length of the capillary}$ ) across the extraction capillary as a result of the shorter capillary length. Incomplete removal of DS<sup>-</sup> was also observed with the other lengths studied.

For the applied voltage study (5, 10, 15 kV), 15 kV resulted in very unstable currents and bubble formation in the multi-capillary assembly. 5 kV produced very slow DS<sup>-</sup> removal that was impractical for routine sample processing. At a voltage application time of 12 min, a long zone of DS<sup>-</sup> monomers was observed during ESI-MS analysis, suggesting an incomplete removal of DS<sup>-</sup>.

The effect of simultaneous pressure and voltage application time (0 – 3 min) after sample loading was then investigated. The total voltage application time was 10 min. DS<sup>-</sup> removal and peptide enrichment were observed for all conditions studied. However, 2 min simultaneous pressure and voltage application resulted in a more stable operating system with minimal current instability. The high removal of DS<sup>-</sup> was also faster with the 2 min

simultaneous pressure and voltage application time, since the end of the sample plug was closer to the interconnect tee where electrokinetic injection of the SDS and the SDS-micelle bound peptide occurred.

#### **4.4.4. Removal of $DS^-$ from digested BSA samples.**

The utility of the proposed method was tested using tryptic digested BSA sample solution. Figure 4.4.4.1 shows the ESI-MS spectra before (A) and after (B) removal of  $DS^-$  from the sample. In Figure 4.4.4.1A, the SDS signals were visible in the spectra and there were strong signals for peptides ( $m/z$  409.9, 509.1, 797.2, 896.2). In Figure 4.4.4.1B, SDS signals were absent in the spectra, suggesting the removal of  $DS^-$  by the proposed approach. The strong signals of peptides observed in 4.4.4.1A were also present 4.4.4.1B, but with a noticeable increase in signal intensity. For example, the signal intensity of  $m/z$  509.1 was  $9.0 \times 10^4$  and  $5.4 \times 10^6$  before and after  $DS^-$  removal, respectively. A 60-fold improvement of the signal was calculated (improvement = quotient of the signals after and before treatment). This was due to the enrichment effect of our device. Moreover, there was significant improvement in the signals for peptides that were significantly suppressed by SDS (see the small signals for peptides from  $m/z$  600 to 1200 in Figure 4.4.4.1A). In addition, peptide signals that were not found in Figure 4.4.4.1A appeared as strong signals in Figure 4.4.4.1B. For example, the signals of peptides and adducts ( $m/z$  947.0, 955.2, 986.3) in Figure 4.4.4.1B were undetected in Figure 4.4.4.1A (see inset in Figure 4.4.4.1B,  $m/z$  940 – 990 was expanded). The observed enrichment factors of signals (= signals after removal/ signal before removal) were 535, 693, and 656 for  $m/z$  947, 955.2, and 986.3, respectively. The peptide %recovery values (= number of peptides before or after removal / number of theoretical peptides x 100%) for BSA in Figure 4.4.4.1A and 4.4.4.1B were 14% and 87%, respectively. Table 4.4.4.1 shows the identified peptides of the prominent peptide signals in Figures 4.4.4.1A and 4.4.4.1B. This clearly indicates the significant improvement of peptide %recovery via the significant

removal of  $\text{DS}^-$  and enrichment of peptides with electrokinetic on-line integrated SDS removal – ESI-MS

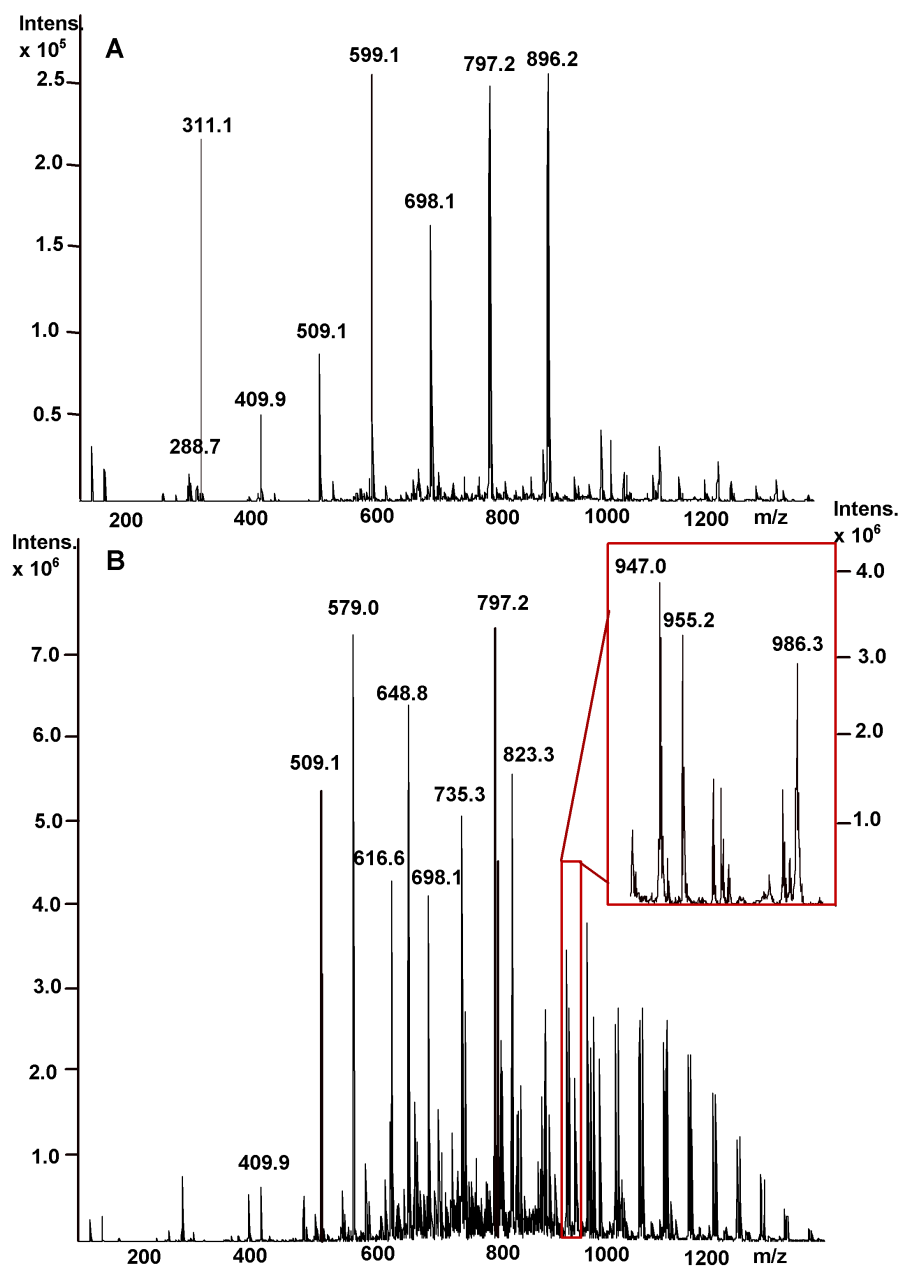


Figure 4.4.4.1. Representative ESI-MS spectra of before (A) and after (B)  $\text{DS}^-$  removal of tryptic digested BSA.

Table 4.4.4.1. Identification of prominent peptide and adduct signals of BSA digests.

<b>m/z in spectra</b>	<b>theoretical M</b>	<b>adducts</b>
Before DS <sup>-</sup> removal (Figure 4.4.4.1A)		
409.9	817.88	$[M + 2H]^{2+}$
509.1	243.29	$[2M + Na]^+$
797.2	1511.72	$[M + 2ACN + 2H]^{2+}$
896.2	1677.88	$[M + 3ACN + 2H]^{2+}$
After DS <sup>-</sup> removal (Figure 4.4.4.1B)		
409.9	817.88	$[M + 2H]^{2+}$
509.1	243.29	$[2M + Na]^+$
616.6	616.6	-
648.8	648.8	-
735.3	1386.48	$[M + 2ACN + 2H]^{2+}$
797.2	1511.72	$[M + 2ACN + 2H]^{2+}$
823.3	2401.65	$[M + 3Na]^{3+}$
Inset (Figure 4.4B)		
947	1851.09	$[M + ACN + 2H]^{2+}$
955.2	922.03	$[M + CH_3OH + H]^+$
986.3	461.46	$[2M + ACN + Na]^+$
		$[M + 2ACN + 2H]^{2+}$

#### **4.5. Conclusion**

An electrokinetic on-line integrated SDS removal – ESI-MS device that was capable of complete removal of  $\text{DS}^-$  and enrichment of peptides was described. The device was composed of pressure and voltage sources, and a multi-capillary assembly. The dimensions of the extraction capillary, which was the most essential part of the multi-capillary assembly, were optimized by varying the i.d. and length. The largest possible i.d. (200  $\mu\text{m}$ ) was used to maximize the electrokinetic introduction of SDS and micelle-bound peptides into the extraction capillary. A short capillary length (5cm) provided a high field strength for faster removal and enrichment. Only a small number of simple experimental parameters, such as voltage applied, voltage application time, and simultaneous pressure and voltage application time, were vital for successful operation. Processing of 1 sample required approximately 20 min, which was faster than other SDS removal methods. A small volume of  $\sim 3.5\ \mu\text{L}$  was loaded onto the device, but a minimum volume of 50  $\mu\text{L}$  was required for introduction into the multi-capillary assembly, which could be attractive when only small volumes of sample are available.

#### 4.6. References

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## Chapter 5

### Conclusion and future directions

The presence of salts and surfactants (i.e. SDS) in sample solutions are detrimental to ESI-MS analysis in bottom-up proteomics. Thus, various sample preparation strategies for desalting and SDS removal prior to ESI-MS have been developed for more efficient elucidation of peptides and proteins. However, most of these clean-up techniques are expensive, laborious, have low throughput, and requires complex devices or experimental set-up. Development of desalting and SDS removal methods that are simple, reproducible, environment-friendly, and require low sample volume are vital for proteomics and biological research.

This thesis project successfully developed one electrokinetic desalting and two SDS removal techniques applied to  $\mu\text{L}$  sample solutions with high salt content and SDS micelles, respectively. The desalting technique (presented in Chapter 2) removed inorganic anions from and retained analytes in the sample vial using a commercial capillary electrophoresis instrument, a capillary filled with an acidic extraction solution, and electric field. The desalting and analyte retention were made possible by controlling the apparent electrophoretic velocities of the inorganic anions and the analytes. The first  $\text{DS}^-$  removal method (presented in Chapter 3) removed  $\text{DS}^-$  from a sample solution containing micellar SDS bound-peptides. The peptides were released from the SDS micelles through micelle-to-solvent stacking, which is the collapse of micelles in the presence of high concentration of organic solvent and reversal of electrophoretic mobilities of the peptides. Effective removal of  $\text{DS}^-$  from the sample solution resulted and the method was successfully applied to digested proteins. Both the desalting and the first  $\text{DS}^-$  removal techniques were performed offline and the sample solution after the removal were directly infused to the ESI-MS instrument for analysis. The



second  $\text{DS}^-$  removal method (presented in Chapter 4) was interfaced to the ESI-MS instrument using a multi-capillary assembly developed in our laboratory. Another important feature of this method, aside from the efficient removal of  $\text{DS}^-$ , was the significant MS signal improvement of the peptide due to the enrichment of the peptides inside the assembly. The ease of assembly and minimal sample volume requirement also made this method attractive.

In this PhD thesis, the attempt to develop relatively cheap, environmental-friendly, and high throughput strategies for desalting and SDS removal were successful. However, this work is not without limitations and certainly has a lot of room for improvement. Firstly, the desalting method, which was applied to samples of 1000 mg/L chloride concentration in 45 min, could be further improved to be able to accommodate higher conductivity sample solutions in short processing times. A bench-type capillary electrophoresis instrument and power supply designed to handle and accommodate high voltage applications and current production would be a good alternative for desalting high conductivity sample solutions. Secondly, the limitation of the offline  $\text{DS}^-$  removal method, which is the improvement of MS signal of the analytes, was circumvented by the development of the multi-capillary assembly interfaced to the ESI-MS instrument. Stability of the assembly had been the main issue in this part of the work. A more robust design of the assembly is strongly suggested to improve the reproducibility of the method. In addition, automation would be a great direction for this online method for better sample throughput.